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Metabolic Reprogramming of Pancreatic Ductal Adenocarcinoma Cells in Response to Chronic Low pH Stress

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Metabolic Reprogramming of Pancreatic Ductal Adenocarcinoma Cells in Response to Chronic Low pH Stress

by

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Pancreatic ductal adenocarcinoma (PDAC) is among the most lethal of all cancers with a 5-year survival rate of only 8.2%. This is because PDAC is diagnosed in its advanced stages and is characterized by radio and chemotherapy resistance. Aggressiveness of PDAC tumors is attributed to its high metabolic phenotype, which is characterized by increased glycolysis rate and lactate secretion, while oxidative metabolism is reduced. These metabolic features are required to fulfill the biosynthetic demands of proliferating PDAC cells. However, this increase in metabolic activity results in acidification of the extracellular space because the dense fibrotic stroma of PDAC tumors limits venting of protons into the vasculature thereby creating a chronic low pH microenvironment. Little is known regarding the physiology and metabolism of cancer cells enduring chronic low pH exposure.

To demonstrate effects of low pH, PDAC cells were cultured in low pH 6.9~7.0 to establish chronic low pH as it occurs in tumors. These cells were compared to cells in physiological pH of 7.4, which is also the pH of cell culture, in order to evaluate physiological differences between these pH values. In these experiments, it was observed that cells in low pH have reduced clonogenic capacity and undergo a metabolic shift to oxidative metabolism that is supported by an increase in glutamine uptake. These observations exhibit a robust contrast to PDAC cells in control pH conditions that are highly glycolytic. Furthermore, in low pH there is increased

transcription of the GOT1 enzyme, which mediates metabolic flux through the non-canonical glutamine metabolic pathway that allows synthesis of other metabolic substrates from glutamine. Upon shRNA-mediated depletion of GOT1, survival of PDAC cells in low pH was significantly impaired due to increase in ROS to cytotoxic levels. However, supplementing transfected clones with GOT1 metabolic product, oxaloacetate, resulted in growth rescue and reduction in ROS levels. Thus, in chronic low pH stress PDAC cells up-regulate non-canonical glutamine metabolism through increased transcription of GOT1, which allows PDAC cells to generate energy and metabolic co-factors to suppress cytotoxic ROS levels. Low pH is a universal feature of the PDAC tumor microenvironment and further dissection of metabolic adaptations to microenvironment conditions will result in more effective therapy for PDAC.

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6b,6C Non-canonical glutamine metabolism regulates ROS levels in chronic low ph

Chapter 3

Figures

1 Hypothetic metabolic-pH relationship in PDAC tumors

List of Abbreviations

(aq)	Aqueous
(g)	Gas
(l)	Liquid
[^{3}H]	Titriated Hydrogen in Glucose/Glutamine
2PG	2-Phospho-Glycerate
3PG	3-Phospho-Glycerate
A^-	Conjugate Base
α-Keto	Alpha-Ketoglutarate
α-KG	Alpha-Ketoglutarate
α-SMA	Alpha-Smooth Muscle
Ac-CoA	Acetyl-Coenzyme A
ACLY	ATP citrate lyase
ADP	Adenosine Diphosphate
AE	Anion Exchanger
AKT	Serine/Threonine Kinase
ANOVA	Analysis of Variance
AOA	Aminooxiacetic Acid
AQP	Aquaporin
Asp	Aspartate
ATG5	Autophagy Related Gene 5
ATM	Ataxia Telangiectasia Mutated
ATM	Atmospheric Pressure
ATP	Adenosine Triphosphate
ATPase	ATP hydrolyzing Enzyme
B-Ox	Beta-oxidation
BAX	BCL2 Associated X Protein
BMI	Body Mass Index
Brackets [ ]	Denote Concentration
BRCA1	BRCA1, DNA Repair Associated, isoform 1
BRCA2	BRCA1, DNA Repair Associated, isoform 2
BT	Bicarbonate Transporter
^{13}C	Carbon-13, Natural Isotope
c-MYC	Myelocytomatosis oncogene
CA	Carbonic Anhydrase
CAII	Carbonic Anhydrase 2
CAIX	Carbonic Anhydrase 9
CAXII	Carbonic Anhydrase 12
CD4	Cluster of Differentiation 4
CDCFDA	5-(and-6)-Carboxy-2',7'-Dichlorofluorescein Diacetate
CDK2	Cyclin Dependent Kinase 2

CDK4	Cyclin Dependent Kinase 4
CDKN2A	Cyclin Dependent Kinase Inhibitor 2A
cDNA	Complementary DNA
Cit	Citrate
Cl^-	Chloride Ion
CO2	Carbon Dioxide
CoA	Coenzyme A
COX	Cyclooxygenase
COX2	Cytochrome Oxidase Subunit 2
CS	Citrate Synthase
CTLA4	Cytotoxic T-Lymphocyte Associated Protein 4
Cyc	Cyclin (figure)
DCFDA	2',7' –Dichlorofluorescin Diacetate
DHAP	Dihydroxiacetate-Phosphate
DMEM	Dulbecco Modified Eagle Medium
E3	Enzyme 3 in Ubiquitin Ligation Process
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechin Gallate
EGF	Epithelial Growth Factor
EGFR	Epithelial Growth Factor Receptor
ENO1	Enolase, Isoform 1
ENO2	Enolase, Isoform 2
ErbB2	Receptor tyrosine-protein kinase erbB-2
ErbB3	Receptor tyrosine-protein kinase erbB-3
ERK	Extracellular Singal Regulated Kinase
ETC	Electron Transport Chain
F-6-P	Fructose-6-Phosphate
FA	Fatty Acid
FAD^+	Oxidized Flavin Adenine Dinucleotide
$FADH_2$	Reduced Nicotinamide adenine dinucleotide
FANCC	Fanconi Anemia Complementation Group C
FANCG	Fanconi Anemia Complementation Group G
FAO	Fatty Acid Oxydation
FASN	Fatty Acid Synthase
FBP	Fructose-1,6-Bisphosphate
FBP	Fructose-1,6-Bisphosphate
FBS	Fetal Bobine Serum
FDG	Fludeoxyglucose
FGF	Fibroblast Growth Factor
G-6-P	Glucose-6-Phosphate
G1	Growth 1 Stage of Cell Cycle
G12	Glycine 12

G12D	Glysine-12 to Aspartate Mutation
G12V	Glysine-12 to Valne Mutation
G13	Glycine 13
G13D	Glysine-13- to Aspartate Mutation
G2	Growth 2 Stage of Cell Cycle
G2A	G2 Accumulation Protein
G3P	Glyceraldehyde-3-Phosphate
G6P	Glucose-6-Phosphate
G6PD	Glucose-6-phosphate dehydrogenase
GAMT	Guanidinoacetate N-Methyltransferase
GAP	GTPase Activating Protein
GDP	Guanosine Diphosphate
GEF	Guanine Nucleotide Exchange Factor
GF	Growth Factor
GFPT1	Glutamine-Fructose-6-Phosphate Transaminase 1
Gln	Glutamine
GLS	Glutamate Synthase
GLS1	Glutamate Synthase, Isoform 1
GLS2	Glutamate Synthase, Isoform 2
Glu	Glutamate
Gluc	Glucose
GLUD	Glutamate Dehydrogenase
GLUT1	Glucose Transporter, Isoform 1
GLUT3	Glucose Transporter, Isoform 3
GLUT4	Glucose Transporter, Isoform 4
GOT1	Glutamic-oxaloacetic transaminase 1, Cytoplasmic Isoform
GOT2	Glutamic-oxaloacetic transaminase 2, Mitochondrial Isoform
GPCR	G-protein Coupled Receptor
GPR4	G Protein-Coupled Receptor 4
GPX	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
GTP	Guanosine Triphosphate
GTPase	GTP Hydrolase enzymes
H^+	Hydrogen Ion
H_2CO_3	Carbonic Acid
H_2O	Water
H_2O_2	Hydrogen Peroxide
H_3O^+	Hydronium Ion
HA	Hyaluronic acid
HA	Bronsted-Lowry Acid
HB	Conjugate Acid

Hb	Hemoglobin
HBP	Hexosamine Biosynthesis Pathway
HCl	Hydrochloric Acid
HCO_3^-	Carbonate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF	Hypoxia Inducible Factor (Complex)
HIF1α	Hypoxia Inducible Factor 1 Alpha
HIF1β	Hypoxia Inducible Factor 1 Beta
HK	Hexokinase
HK2	Hexokinase II
HRE	Hypoxia Response Elements
HSP90	Heat Shock Protein 90
IC50	Inhibitory Concentration 50%
IDH	Isocitrate Dehydrogenase
IDH1	Isocitrate Dehydrogenase, Isoform 1
IDH2	Isocitrate Dehydrogenase, Isoform 2
IFNϒ	Interferon Gamma
IFP	Interstitial Fluid Pressure
IGFR	Insuline-like Growth Factor Receptor
IL-2	Interleukin 2
Isocit	Isocitrate
K_a	Acid Dissociation Constant
K_H	Henry's Law of Gases Equilibrium Constant
K_w	The water ionization constant
LAG3	Lymphocyte Activation Gene 3
LC-MS/MS	Liquid Cromatography Coupled with Tandem Mass Spectrometry
LCMS	Liquid Cromatography Coupled with Tandem Mass Spectrometry
LDHA	Lactate Dehydrogenase A
LDHB	Lactate Dehydrogenase B
M	Mitosis Stage Cell Cycle
MAPK	Mitogen Activated Protein Kinase
MCT1	Monocarboxylate Transporter, Isoform 1
MCT4	Monocarboxylate Transporter, Isoform 4
MDH1	Malate Dehydrogenase 1
MDH2	Malate Dehydrogenase 2
MDM2	Mouse double minute 2 homolog
ME1	Malic Enzyme, Isoform 1
ME2	Malic Enzyme, Isoform 2
mmHg	Millimeter of Mercury
mRNA	Messenger RNA
mTOR	Mammalian Target of Rapamycin

MTT	Micro culture Tetrazolium Assay
MUC1	Mucin 1
N (figure)	Nucleus
NAC	N-acetyl Cysteine
NAD^+	Oxidized Nicotinamide adenine dinucleotide
NADH	Reduced Nicotinamide adenine dinucleotide
$NADP^+$	Oxidized Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
$NaHCO_3$	Sodium Bicarbonate
NBC	Sodium Bicarbonate Transporter
NEAA	Non-essential Amino Acids
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear Factor Kappa-light-chain-enhancer of Activated B cells
NHE	Sodium Hydrogen Exchanger
NHE1	Sodium Hydrogen Exchanger 1
NK	Natural Killer Cell
NOX1	NADPH Oxidase 1
NOX2	NADPH Oxidase 2
NOX3	NADPH Oxidase 3
NRF1	Nuclear Respiratory Factor 1
O-GlcNAc	O-linked N-Acetyl Glucosamine
O2	Oxygen (gas)
OAA	Oxaloacetic Acid
OGA	O-GlcNAcas
OGDC	Oxoglutarate Dehydrogenase Complex
OGR1	Ovarian cancer G protein-coupled receptor 1
OGT	O-GlcNAc transferase
OH^-	Hydroxide Ion
$p16^{INK4A}$	p16 encoded by Cyclin Dependent Kinase Inhibitor 2A
$p19^{ARF}$	p19 Alternative Reading Frame
p300	p300 Protein
pAKT	Phosphorylated AKT
PALB2	Partner and localizer of BRCA, isoform 2
PanIN	Pancreatic Intraepithelial Neoplasm
PBS	Phosphate-Buffered Saline
PC	Pyruvate carboxylase
PCK	Phosphoenolpyruvate carboxykinase
pCO_2	Partial Carbon Dioxide Gas Pressure
PD1	Programmed Cell Death Protein 1
PDAC	Pancreatic Ductal Adenocarcinoma
PDGF	Platelet Derived Growth Factor
PDH	Pyruvate Dehydrogenase (Complex)
PDK	PDH Kinase

PDK1	Pyruvate Dehydrogenase Kinase
PDP	PDH Phosphatase
PDX1	Pancreatic And Duodenal Homeobox 1
PEP	Phosphoenol Pyruvate
PET	Positron Emission Tomography
PFK	Phosphofructokinase
PGK1	Phosphglycerate Kinase 1
PGM	Phosphoglycerate Mutase
pH	Potential of Hydrogen
PHD	Propyl Hydroxylase Enzymes
pH_e	External pH
pH_i	Internal/Cytosolic pH
pI	Isoelectric Point
P_i	Phosphate group
PI3K	Phosphatidylinositol-4, 5-bisphosphate 3-kinase
PK	Pyruvate Kinase
pK_a	Partial Acid Dissociation Constant
PKM1	Pyruvate Kinase M1 Isoform
PKM2	Pyruvate Kinase M2 Isoform
pO_2	Partial Oxygen Pressure
pOH	Potential of Hydroxide
PPI	Proton Pump Inhibitor
PPP	Pentose Phosphate Pathway
pRB	Phosphorylated Retinoblastoma
PSC	Pancreatic Stellate Cell
PTEN	Phosphatase and tensin homolog
PUMA	The p53 upregulated modulator of apoptosis
Pyr	Pyruvate
Q61	Glutamine 61
Q61H	Glutamine-61 to Histidine Mutation
R273H	Arginine 273 to Histidine Mutation
RAD-GTP	GTP Bound KRAS
RAF	RAF Family of Proto-Oncogene Serine/Threonine-Protein Kinase
RAL	Ras Related Protein
RAS	Ras Family of Protein GTPases
RAS-GDP	GDP Bound KRAS
RB	Retinoblastoma
RBC	Red Blood Cell
RCC	Renal Cell Carcinoma
ROS	Reactive Oxygen Species
RPE	Ribulose-phosphate 3-epimerase
RPIA	Ribose-5-phosphate isomerase

RT-qPCR	Real-Time Quantitative Polymerase Chain Reaction
S	DNA Synthesis Stage of Cell Cycle
S.E.M.	Standard Error of Mean
SCO2	Cytochrome C Oxidase Assembly Protein
SDS	Sodium Dodecyl Sulfate
SEER	Surveillance, Epidemiology, and End Results Program
shGOT1	Short-hairpin RNA Targeting GOT1
shGOT1a	shRNA vector 34784
shGOT1b	shRNA vector 34785
SHH	Sonic Hedgehog
shRNA	Short-hairpin RNA
shScr	Short-hairpin Scramble RNA
SLC1A5	Solute Carrier 1A5
SLC26	Solute Carrier 26
SLC4	Solute Carrier 4
SMAD4	SMAD Family Member 4
SOD1	Superoxide Disumutase 1
SOD2	Superoxide Disumutase 2
SOX9	SRY-Box 9
STR	Short Tandem Repeat
Succ-CoA	Succinyl-CoA
T-reg	Regulatory T-Cell
TAM	Tumor Associated Macrophage
TCA	Tri-carboxylic Acid
TDAG8	T-cell death-associated gene 8
TGFB	Tumor Growth Factor Beta
TIGAR	*TP53*-inducible glycolysis and apoptosis regulator
TIM3	T-cell immunoglobulin and mucin-domain containing-3
TME	Tumor Microenvironment
TNF	Tumor Necrosis Factor
TP53	Tumor Protein P53
TSC	Tuberous sclerosis complex
Ub	Ubiquitin (figure)
UDP-GlcNAc	Uridine diphosphate N-acetylglucosamine
v-ATPase	Vacuolar ATPase
VHL	von-Hippel Lindau E3 Ligase

Chapter 1

Introduction:

The Evolution Of PDAC And Its Acidic Tumor Microenvironment

Incidence of Pancreatic Ductal Adenocarcinoma

According to the American Cancer society, the estimate of new cases of pancreatic cancer in 2017 is 53,670 and the number of estimated deaths is 43,090 [1, 2]. Needless to say, the 5-year survival rate is also very low at 8.2%, **figure 1** [1, 2]. These grim statistics have remained virtually unchanged over the past two decades. For this reason, a diagnosis of pancreatic ductal adenocarcinoma (PDAC), the most common type of pancreatic cancer representing 90% of all pancreatic cancers, is synonymous of a death sentence as it carries one of the most dismal prognoses in all of medicine [3]. Poor disease outcome can be attributed to the lack of specific symptoms and limitation in diagnostic methods allowing PDAC to elude detection during its formative stages; as a result, PDAC is typically diagnosed in its advanced stages [4]. Currently, the only reasonable hope of cure is through surgical resection, which in itself is a life-threatening procedure, but rarely a curative end point is achieved due to disease spread and recurrence [5]. Non-invasive procedures include radiation therapy and chemotherapy; however, improvement of long-term survival is minimal due to pronounced PDAC therapy resistance [6]. Thus, most treatments are palliative with the goal of improving the quality of life in patients [6]. Alarmingly, the incidence of PDAC is expected to double within the next 15 years [6]. For this reason, there is desperate need to understand the complex biology of PDAC carcinogenesis in order to identify more effective therapeutic approaches and improve patient survival.

Development of Pancreatic Ductal Adenocarcinoma

Acquisition of Oncogenic Mutations

A basic tenet of Natural Selection is the constant occurrence of purposeless mutations that increase genetic variability on which selection forces act. For a mutation to occur, a complete cell division cycle must take place. Human DNA polymerases have an error at a rate of $1x10^{-9}$ per base on each replication round [7-9]. Therefore, statistically speaking, three single nucleotide variants occur per replication cycle of somatic cells. Considering the human haploid genome is $3x10^{9}$ base pairs long and that the number of cells in an adult body is approximately 10^{14}, mutations are likely to occur at any time [8]. However, mathematical models predict that the emergence of new strategies (phenotypes) in individual cells during tissue turnover does not improve fitness and cancer requires more steps [10, 11]. Furthermore, not all cells in the body are actively replicating this includes acinar and ductal cells of the adult pancreas where PDAC originates [12, 13]. Thus, by simple chance alone, the incidence of PDAC is rare, but if PDAC is to develop the initial mutation must be fixed to the normal tissue landscape in order to hide from pathologic response such as tissue growth constraints and immune responses that would lead to senescence and/or apoptosis. This will allow the pre-malignant phenotype to proliferate giving more opportunities for its clones to gain additional genomic instability that will lead to cancer, i.e. PDAC signature. Perhaps, this explains the time frame between initial mutation, which occurs at minimum two decades, and PDAC diagnosis [13]. The median age for diagnosis of PDAC is 71 and 75% of cases are diagnosed between the ages of 55 and 84 [2, 3, 13]. Recent statistical analyses of various cancer types, including PDAC, calculated high risk due to a strong correlation with aging and the predicted number of normal stem cell divisions [14, 15]. However, there is much debate to describe links for PDAC induction due to intrinsic factors, carcinogens, and non-cancerous pathophysiology of cancer.

Intrinsic factors occur because of chance events such as failure in genetic replication, but this event is very unlikely in healthy patients. The most common intrinsic factors that increase PDAC risk are familial traits where gene variants involved in DNA double-strand break repair such as BRCA1, BRCA2, PALB2, FANCC, FANCG, and ATM [16]. Understandably, defective DNA repair mechanism would increase genetic instability and chances for somatic mutations to occur. Familial variants affecting tumor suppressors also have robust incidence with PDAC. For example, germline mutations to *CDKN2A* that encodes two proteins that regulate cell cycle progression these are $p16^{INK4A}$ and $p19^{ARF}$ [16]. Carcinogens that damage the genome or alter metabolic functions, as well as, tissue inflammation, cell turnover due to idiopathic diseases, and organ specific disease all account for extrinsic factors of high PDAC risk [17]. It has been demonstrated extensively that continuous exposure to pro-inflammatory agents increases ROS levels that promote DNA damage [18]. Inflammation of the pancreas, also known as pancreatitis, caused by alcohol abuse and smoking is associated high PDAC risk [19, 20]. Similarly, patients diagnosed with PDAC are often diagnosed with diabetes type II (hyperinsulemia) and are classified as obese; both of these conditions are associated with chronic inflammation of the pancreas [21]. Although it is unclear the mechanism of cooperation between genomic alterations and tissue damage, both of these cause initial events leading to PDAC. In the context of PDAC evolution, it is likely that the initial mutation occurs randomly, becomes fixed to tissue landscape, and awaits for tissue damage to induce other genomic alterations and evolve to PDAC [10, 14]. Thus, one could argue that the initial mutation is caused intrinsically or by exposure to carcinogens and life style choices such as diet and alcohol abuse will increase pancreatic burden paving the way for somatic evolution of PDAC. Therefore, healthy status and normal function of the pancreas is critical in holding back PDAC. Table 1A and Table 1B highlight intrinsic and extrinsic PDAC risk factors.

Pancreatic Ductal Cell Function

The pancreas is a glandular organ of the digestive and endocrine systems located in the abdominal cavity behind the stomach [22]. The histological features of the pancreas are easily distinguishable under the microscope. Pancreas tissue with endocrine role is arranged in clusters called pancreatic islets and constitutes about 1 to 2% of the total pancreas volume [22]. The critical metabolic hormones for metabolic response, insulin and glucagon, are produced by beta and alpha cells, respectively, in pancreatic islets [5]. The rest of the pancreas functions as part of the exocrine digestive system composed of exocrine and ductal epithelium [22]. Acinar exocrine cells of the pancreas, form clusters called acini, for the berry-like sac architecture [5]. This hollow structure formed by acinar cells converges with epithelial cells of the pancreas to form a ductal system designed for the delivery of digestive enzymes to the duodenum [5, 22]. The enzymes secreted by acini are lipase, proteases, and nucleases; while the ductal cells secrete large amounts of sodium bicarbonate ($NaHCO_3$) [22, 23].

Pancreatic cancers develop 95% of the time from exocrine cells and most commonly from ductal cells 85-95% (PDAC), while cancer from acinar cells and endocrine both represent less than 5% [22]. This is likely because more progenitor cells are needed to replace the dispensable epithelial cells lining the pancreatic ducts because ductal epithelium is exposed to more stress and thus progenitor cells undergo more replication cycles increasing the chance for acquiring mutations. Some ambiguity in PDAC origin exists because acinar cells are known to undergo acinar-ductal metaplasia in pre-malignant lesions through increased expression of KRAS and pancreatic progenitor cell transcription factors SOX9 and PDX1 [24]. Interestingly, pancreatic cancers of exocrine origin are much more aggressive than pancreatic

endocrine [25]. Thus, the function of pancreas exocrine and its condition in pathologic circumstances must be addressed to learn more about PDAC initiation.

In normal conditions the pancreatic ductal cells secrete up to 140mM of sodium bicarbonate, which is useful to neutralize the acid chime that enters the duodenum [23]. Interesting research on inhibition of bicarbonate release by ductal cells shows to be reduced upon enzyme release by acinar cells while acinar secretion of chloride and ATP has been demonstrated to stimulate ductal bicarbonate secretion [23]. Furthermore, recent findings show that acinar exocytosis causes a significant acid load into the ductal cavity thereby decreasing the pH by up to 1 unit [26]. Normally the pH in duct fluid is 8, **figure 2** [26]. Therefore, pH control by ductal cells is critical for tissue homeostasis, as decease in pH has been shown to induce inflammation [23]. This has been demonstrated in animal models where induction of pancreatitis by cerulein treatment lowers the pH of ductal fluid [23, 27]. Similar findings have been noticed in drug treatments, acid injections to the pancreas in experimental pancreatitis mouse models, and in mutations affecting mitochondrial function that cause lactic acidosis [28]. Interestingly, ethanol exposure has been shown to decrease blood flow to the pancreas by up to 60% causing hypoxic response and drop in pancreatic tissue extracellular pH (pH_e) to 7.1 [29]. This study highlights the role of the pancreatic ductal cells in blood pH homeostasis by removing CO_2 (metabolic acid) and converting it to bicarbonate that is then used to regulate the pH of ductal fluid. Furthermore, idiopathic diseases affecting blood flow may damage the pancreas indirectly by altering pH dynamics. Interestingly, approximately 75% of PDAC tumors occur in the head of the pancreas where it links to the duodenum. Therefore, bicarbonate secretion from ductal cells is critical to buffer the pH from stomach acids and acinar secretions. These observations show how critical bicarbonate secretions from ductal cells are for pH regulation for normal exocrine tissue

function, as lowering of the pH increases risk of pancreatitis, which in turn increases risk for PDAC greatly [30].

Pancreatic Ductal Adenocarcinoma as an Evolutionary Process

Solid tumors, such as PDAC, appear to progress in a stepwise manner. The first step is acquisition of driving mutations, followed by clonal expansion from an initial malignant cell, and third is local invasion and metastasis. Intriguingly enough, progression of PDAC drastically affects the physical features of its tumor microenvironment (TME) characterized by hypoxia, acidosis, and high interstitial pressure [31]. However, the harsh conditions in the TME do not appear to oppose carcinogenesis, instead, it seems to promote fitness of the tumor cells promoting acquisition of phenotypes that promote cancer aggressiveness. Throughout this introductory chapter, I will address carcinogenesis of PDAC as if it were a process subjected to Darwinian evolutionary processes with the goal of instigating novel insights and opportunities for study.

The model of Natural Selection, as proposed by Charles Darwin, is simply defined by the differential rates of survival and reproduction due to differences in phenotype of competing species [32]. These rules impose that the carrier of the fittest phenotype will get to survive, reproduce, and pass on the given trait to offspring. The process of evolution can be categorized by the strategy of adaptability (phenotype) and predictable outcomes allowing us to mathematically model evolution of species populations with the given resources of an ecosystem [32]. Adaptations of these mathematical methods have been used to determine the evolutionary potential of cancer cells [10, 33]. These mathematical models utilize two broad control mechanisms of multicellular organisms, which are tissue development regulators and substrate availability [10, 33, 34]. The former consists of complex growth control mechanisms such

as: cell-cell and cell-ECM (extracellular matrix) interactions, as well as, response to soluble growth factors [35]. Glucose is the universal substrate in catabolic metabolic reactions; thus tissue uptake capacity and basal metabolic uptake can be calculated based on extant literature [10, 33].

Mathematical analysis of normal epithelial tissue dynamics reveals that populations of normal cells have decreased fitness upon reaching tissue equilibrium [10]. Furthermore, evolutionary models show that loss of fitness is independent of growth strategy (phenotype), which means that tissue stability is due to cooperation of co-existing non-competing populations, if more than one population with different survival strategy were to exist within the same tissue [10, 33]. Therefore, each individual cell in epithelial tissue gives away its individual growth potential for normal tissue development and the overall good of the whole organism. This also implies that initial oncogenic mutations will not cause cancer alone, but will rather be fixed into the tissue landscape. Subsequent evolution of cancer is elicited by environmental factors such as inflammation, which will cause clonal expansion of pre-cancerous tissue-fixed cells giving room for acquisition of somatic mutations to further enhance fitness of mutant cells causing a relaxation in growth regulation trough contact inhibition by disrupting to cell-cell or cell-basement membrane interactions [35]. Further adaptations promoting the cancer phenotype are induced by micro-environmental changes such as hypoxia and/or lack of nutrients since cell population growth leads to separation from blood vessels [36]. Thus, mathematical simulation models of cancer evolution are possible if the factors of dynamic progression of cancer are provided through experimental observation [10, 33, 34]. Based on mathematical modeling and experimental observations the evolutionary model of carcinogenesis has 3 defined stages of cancer: initiation, promotion, and progression, **figure 3.**

PDAC carcinogenesis fits an evolutionary model of cancer progression since its genetic signature is well characterized (*KRAS*, *CDKN2A*, *TP53*, *SMAD4*), as well as, characteristics of its TME (hypoxia, acidosis, high interstitial pressure) and survival outcome (activation of Warburg effect metabolism, fibroblast activation, immune evasion) [5, 36, 37]. Thus, in its formative stages PDAC is limited by tissue growth constraints (extrinsic factors), but once the oncogenic phenotype arises further survival is defined by cellular homeostasis and the metabolic adaptations required to sustain it. In the following sections I will address how pancreatic epithelial cells become oncogenic to develop PDAC.

Neoplastic evolution of PDAC

The evolution of malignant cell into PDAC requires several steps that have been histologically characterized by observable pancreatic lesions, also known as pancreatic intraepithelial neoplasms (PanINs). The normal pancreatic ductal epithelium houses low-cuboidal cells lining ducts in a single layer and nuclear atypia is not seen [38]. PanIN-1, is characterized by increased mucin secretion, cell elongation, and observable papillary architecture. PanIN-2, is characterized by nuclear abnormalities such as: nuclear crowding, hyperchromatism, and enlarged nuclei, as well as, some loss of polarity [38]. Cells budding into the lumen, nuclear atypia, and increased mitosis characterize PanIN stage 3 [38]. PDAC diagnosis follows PanIN-3, as it is characterized by invasive growth and marked desmoplasia [38]. PDAC tumors show an increased frequency of mutations in *KRAS*, *CDKN2A*, TP53, and *SMAD4* with increasing PanIN staging [14].

Substantial evidence in PanINs suggests PDAC occurs in a linear progression. Mutant KRAS is expressed in 99% of PanIN-1 and its proportionality increases in high-

grade pancreatic lesions [38]. This indicates fixation of oncogenic KRAS in normal pancreatic epithelium and its selection in pancreatic lesions. Loss of $p16^{INK4A}$ is known to occur most often during PanIN-2 and it shows increased loss in PanIN-3 [38]. Loss of *TP53* and *SMAD4* is known to occur in PanIN-3 lesions and invasive PDAC [38]. The accumulation of these classic features of the PDAC genetic landscape show a pattern of progression that seems to require progressive fixation of these mutations as if these alterations are requirements in evolution of somatic cell to PDAC, i.e. *KRAS→CDKN2A→TP53→SMAD4*. In the following sections, I will address more specifically the stepwise progression of PDAC.

1. *KRAS*

KRAS is a member of the RAS family of small-protein GTPases, whose enzyme function is to hydrolize GTP into GDP and acts as a signal transducer when active in the GTP conformation, **figure 4** [39, 40]. In normal cells KRAS is activated by extracellular growth factors and consequent growth-factor receptor activation. Active KRAS is an important molecule in a plethora of signal transduction pathways, **figure 4** [39, 40]. For this reason it is instrumental in cell to growth, proliferation, as well as, maturation of cells to take on specialized functions (differentiation) [39, 40]. Wild-type KRAS is regulated by guanine exchange factor proteins (GEFs) whose function is to turn off/on by exchanging GDP for GTP [39, 40]. Therefore, KRAS function is regulated by growth factors, GEFs, and GTP/GDP ratio. Most mutations in the *KRAS* gene are believed inhibit GTP hydrolysis thereby becoming constitutively active [39, 40]. *KRAS* is located on the short arm of chromosome 12, and it suffers mutations in codons G12, G13, and Q61 with the most common amino acid substitutions being G12D, G12V, G13D, and Q61H [39, 40]. Oncogenic KRAS expression is nearly ubiquitous in PDAC carcinogenesis with ~99%

expression in PanINs and 95% in tumors [38]. Oncogenic KRAS provides fitness to malignant cells because it mediates uncontrolled proliferation.

Several studies have demonstrated that autocrine epithelial growth factor (EGF) signaling occurs in low-grade PanINs due to overexpression of EGF-family ligand receptors EGFR and ERBB2 [5, 40, 41]. Similarly, EGF signaling results in stimulation of the phosphatidylinositol-3-kinase (PI3K) pathways [41]. Activated KRAS can itself interact with and activate PI3K [40]. PI3K acts on downstream effectors AKT and mTOR (PI3K/AKT/mTOR transduction signaling pathway) and activates metabolic pathways that enhance the cellular rate of macromolecule biosynthesis by increasing membrane expression of nutrient transporters and glycolysis genes [41]. More specifically, AKT stimulates lipid synthesis, while mTOR regulates protein translation [41]. Hypothetically, acute damage to pancreatic epithelium will lead to increase in EGFR in response to injury and to promote tissue regeneration. However, if a cell bearing oncogenic KRAS has been fixed into the pancreatic tissue, increased cell turn over caused by tissue damage will stimulate both EGFR-dependent activation of PI3K/AKT/mTOR pathway and oncogenic-KRAS. In this scenario the mutant cells may expand because of growth factor independency. However, cell division is restrained by cell-cell, cell-basement membrane interactions, and growth cycle checkpoint inhibition [17]. Maintenance of oncogenic KRAS is central for continued uncontrolled growth and increased metabolic rate in advanced disease.

2. *CDKN2A*

The second genomic alteration in the PDAC stepwise progression is the loss of tumor suppressor *CDKN2A*, which encodes for genes $p16^{INK4A}$ and $p19^{ARF}$ because of a shared locus on chromosome 9p [42]. These genes are differentially regulated because

of different reading frames on their first exon [43]. Interestingly, the *CDKN2A* locus is inactivated 90% of the time in PDAC and evidence from mouse models and human data suggests mutations are targeted for $p16^{INK4A}$ [44]. However, larger alterations to *CDKN2A* locus will result in loss of both $p16^{INK4A}$ and $p16^{ARF}$ in an event that can contribute to PDAC through different mechanisms [43]. $p16^{INK4A}$ plays an important role in cell cycle regulation by inhibiting cyclin-dependent kinases CDK2 and CDK4 (cyclinD/CDK2,4 complex), which phosphorylate retinoblastoma protein (pRB) causing its disassociation from E2 Factor (E2F) thereby allowing it to begin transcription of cylcinA and cyclinE [45]. These last two may interact with CDK2 (CyclinA, E/CDK2 complex) inducing passage through G1/S-checkpoint of cell cycle, **figure 5** [45]. Loss of *CDKN2A* gene is commonly seen in medium-grade pancreatic lesions, PanIN-2 [5, 38]. Therefore, this adaptation is likely to favor established oncogenic KRAS mutants by allowing the biosynthetic machinery to make more DNA. Furthermore, induction of *CDKN2A* increases during environmental stress to suppress mitogenic stimulation [45]. The *KRAS*-*CDKN2A* mutations promote DNA synthesis that leads to the genomic instability seen in PanIN2, which may cause DNA sequence errors and/or shortening of telomeres to occur forcing activation of *TP53* gene to transcribe p53 DNA damage response genes and cause senescence or apoptosis. Thus, the genetic alterations in *KRAS* and *CDKN2A*/p16 are likely to cause extensive genomic alterations forcing adaptation to suppress DNA damage response, anti-proliferative, and anti-senescence mechanisms all of which may be regulated by p53.

3. *TP53*

The tumor protein 53 (*TP53*), or p53, is a transcription factor whose function is stimulated in response to DNA damage or stress. p53 activation in normal cells leads to growth arrest or apoptosis to maintain genome integrity; hence, its proclamation as

guardian of the genome [46]. *TP53* is somatically mutated, approximately, in 85% of all PDACs, with nearly 66% of mutations targeting its DNA binding domain [38]. The role of p53 in protecting genome integrity of pancreatic cells in preventing PDAC, as demonstrated by familial PDAC that is induced by is loss of gene ataxia telangiectasia mutation (ATM) whose function in cells is to relay stress signals to p53; loss of p53 signaling axis leads to high risk and induction of PDAC [47]. Dysfunctional p53 would not conduct G1/S and G2/M checkpoints or respond to telomere shortening [46]. Thus, absence of p53 response to genomic damage will result in uncontrolled proliferation. Mutations in *TP53* gene correlate with significant features of dysplasia in PanIN-3 [38]. Absence of p53 improves fitness of cells bearing oncogenic KRAS and *CDKN2A*, as the removal of mitosis breaks will result in clonal expansion leading to loss of polarity diminishing growth constraints due to cell-cell and cell-basal membrane interactions. Furthermore, increased rate of mutations will likely select for a new genotype capable to survive in emerging microenvironment stress factors. **Figure 6** highlights the p53 response in normal cells.

4. *SMAD4*

SMAD4 function is lost in approximately 55% of PDACs and contributes to PDAC progression due to inactivation of TGFβ-mediated growth inhibition, which blocks G1/S cell cycle transition [38, 48]. Interestingly, the TGFβ pathway plays an inhibitory role in PanIN-1 and PanIN-2, but supports progression to PDAC in PanIN-3 role possibly because it promotes paracrine signaling leading to activation of pancreatic stellate cells (PSCs) that cause tissue desmoplasia [49]. Indeed, loss of *SMAD4* shows the predisposition of PDAC to form a strong desmoplastic reaction because familial loss of *SMAD4* does not predispose to PDAC [50]. However, if familial *SMAD4* mutation is concomitant with PDAC, the cancer is more aggressive and highly fibrotic [50]. Deletion

of *SMAD4* is a late event in PanIn3 progression to PDAC and is likely a progression allele in PDAC. Nevertheless, loss of function of *SMAD4* decreases ability to restrict unrestricted proliferation and as a result it would lead to increase in fitness of PDAC cells.

Features of Pancreatic Ductal Adenocarcinoma

Genetic expression analyses of PDAC progression in human samples suggests progression of PanIN-1 to PDAC takes approximately 12 years [51]. The time between PanIN lesions and PDAC is not well defined. However, experimental evidence suggests a 3-step process clonal transformation, clonal expansion, and invasion. First ductal cells require an oncogenic mutation (*KRAS*) (transformation), second the mutant cells need to become the dominant phenotype in the tissue (clonal expansion), and lastly intrinsic mechanisms of tumor suppression need be removed through loss of tumor suppressor function (*CDKN2A* and *TP53*) (invasion). Movement of the transformed cell population from the pancreatic ducts into the ductal lumen and developing a stroma characterized by increased desmoplasia and further clonal expansion; these are the histological features of PDAC [38]. Stochastic growth follows initial spread of malignant cells into the pancreas, as the cancer population faces growth and environmental challenges [52]. Further progression of cancer is not immediate as evidenced in genetic expression analyses suggesting progression form early PDAC to metastatic PDAC takes nearly 7 years, possibly because of tissue instability and immune response. During this time, PDAC cells generate a dynamic and complex TME characterized by infiltration of various cell types, extensive extracellular matrix (ECM), as well as, restricted nutrient and oxygen availability [37]. Thus, the TME of the PDAC tumor imposes potent selection forces to shape ongoing adaptation and promote clonal expansion of initial populating

cells, which in turn will increase cellular heterogeneity of cancer cell population. Fitness of cancer cells must be enhanced trough genetic, epigenetic and phenotypic alterations in order to outcompete the host.

The time elapsed between gain of metastatic potential and the time of death is predicted to be nearly 3 years [51]. PDAC diagnoses are assigned four stages: stage 0 (non-invasive), stage 1 (invasion throughout pancreas), stage 2 (lymphatic invasion), stage 3 (invasion to nerves and blood vessels), and stage 4 (metastatic) [3]. Alarmingly, nearly 80% of all cases are found to be invasive with 50% of these bearing metastatic sites [2, 3]. It has been observed that metastasis is an early event in PDAC [53]; thus, tumors do not follow this staged linear progression to metastasize. It is more likely that a random wave of alterations improve fitness of cells in primary site giving rise to cells with metastatic potential. Indeed, genetic expression analyses indicate tumor heterogeneity derives autonomous and non-autonomous cell population. In terms of tumor evolution this means that cancer cells in the primary tumor site must become fixated to the tumor tissue landscape. Once a landscape threshold is reached, cancer cells will be obligated to undergo further selection to gain autonomy from primary tumor constraints the result is gaining the ability to metastasize. Similarly, the same selection process is likely to induce formation of cancer stem cells, which is another survival strategy. This notion is supported through several lines of evidence such as the fact that 99% of metastatic attempts fail as cells cannot survive more than 24 hours [54]. There is also no genetic signature for metastases, but metastasis is favored by acquisition of pre-malignant mutations (*KRAS, CDKN2A, TP53, and SMAD4*) [55]. Lastly, mathematical models also agree that adaptation to microenvironment before metastasis requires 5-10 years [51]. **Figure 7** highlights the progression of PDAC.

PDAC cancer cells exponentially increase substrate uptake, as demonstrated by [^{18}F]fluorodeoxyglucose (FDG) positron emission tomography (PET) imaging of human tumors, which shows significantly increased glucose uptake of tumors compared to normal adjacent tissue [36, 56]. The metabolic shift to increase aerobic glycolysis and limitation of mitochondrial oxidative phosphorylation, also known as the Warburg effect, is a universal feature of solid tumors since increase in glucose uptake is up to 1000 times more than normal cells [34, 36, 56]. Thus, FDG-PET scans are conducted to diagnose PDAC and for evaluation of therapy response of primary site and metastases. This behavior of cancer cells is logical because cells need biomass to proliferate to achieve this metabolic output is maximized [36]. A caveat of increased metabolism is excess production of metabolic acids. Interestingly, cancer cells exhibit a unique pH gradient in which the internal pH (pHi) is alkaline raging from pH 7.3-7.6 and an acidic extracellular pH ranging from pH 6.5-7.0. Experimental evidence shows that an alkaline pHi enhances metabolic rate, but decreased in pHi limits proliferation and survival of cancer cells [57]. In evolutionary terms, this means that survival/fixation of cancer cells in primary tumor landscape and further progression exclusively depends on pH regulation. In the following sections I will address the emergence of altered metabolism in PDAC and evolution to dysregulated pH dynamics because of Warburg effect metabolism.

Desmoplasia a Hallmark of PDAC

The response to heal tissue damage in the pancreas is particularly strong, as evidenced through histological observations in the pancreas of patients with chronic pancreatitis [38, 58]. Activation of fibroblasts leads to deposition of fibers during wound healing response; this is an evolutionarily conserved response to support the multicellular state of higher organisms [59]. Regulation of fibroblasts is coordinated largely through the transforming growth factor beta, TGFβ, signaling [59]. Other features

of wound healing processes include immune suppression, remodeling of the ECM, and increase of epithelial growth factors in order to recover cells lost through injury [60]. The role of increased stromal fibrosis in PDAC carcinogenesis is unchallenged as it enhances immune suppression, drug resistance, and promotes hypoxia—a feature associated with emergence of metastatic cells [61, 62]. Tumor cells activate quiescent pancreatic stellate cells (PSCs) through paracrine signaling from secretion of platelet derived growth factor (PDGF), TGFβ, and sonic hedgehog [61]. Upon activation, quiescent PSCs differentiate into alpha-smooth muscle actin myofibroblasts (αSMAs) allowing for further activation through autocrine signaling by PDGF, fibroblast growth factor (FGF), and TGFβ [61]. Unlike normal tissue where fibroblast activation is inhibited upon wound healing, in cancer PSCs are constitutively activated through cancer cells paracrine signaling. Thereby resulting in continuous deposition of fibers in the tumor stroma.

Extensive evidence shows that PDAC cannot develop without activation of the desmoplastic stroma [61]. As evidenced by pharmacological inhibition of PSC activation in mice resulting in stromal collapse, development of smaller tumors, and overall more effective response to therapy [37]. Similar results were produced through inhibition of sonic hedgehog (SHH) signaling and upon depletion of enzymes responsible for hyaluronic acid (HA) deposition—a major component of PDAC tumor stroma [37, 61]. Contrastingly, it has also been demonstrated that depletion of carcinoma-associated fibroblasts and fibrosis through deletion of αSMA myofibroblasts themselves or SHH induces immunosuppression and accelerates PDAC progression and reduces survival [37]. These observations suggest that distinct components of the PSCs secretome have different properties in promoting or restraining tumor growth. The same logic can be applied for other tumor-stroma resident cells as well, such as macrophages whose

polarization as M1 leads to anti-tumorigenic response and M2 polarization leads to a pro-tumorigenic response [63]. Interestingly, these opposite properties may have a spatially and timely effects on selection of cancer cell populations, as this would lead to loss of a clonal population and replacement by a fitter clone. These waves of growth are likely to contribute to stochastic evolution of PDAC tumor.

The PDAC stroma ECM is rich in HA, fibrillar collagens, and osteonectinn; collectively, these molecules make the dense stroma that functions as a physical barrier shielding the tumor [61]. Consequentially, increased fibrosis results in poor diffusion of oxygen to cancer cells resulting in wide spread hypoxia throughout the tumor [37]. Furthermore, HA is a negatively charged glycosaminoglycan that binds large amounts of water molecules leading to increased interstitial fluid pressure (IFP) [64]. Pathologic IFP occurs because of increased HA in stroma leading to swelling and collapse of the tissue vasculature thereby limiting oxygen diffusion [65]. It may seem detrimental but restriction of metabolic substrates oxygen and glucose would be a strong selection force for survival pathways such as autophagy, pinocytosis, endocytosis, as well as, metabolic reprogramming to compensate for glucose such as glutamine uptake [61, 62, 64-66]. Glucose diffuses radially 280μm away from blood vessels where as blood only diffuse 200 μm; therefore, hypoxia posses a powerful selective force for survival in highly fibrotic tissue [10, 36]. Hypoxic TME is a classic feature of PDAC tumors, cells adapt to hypoxia through metabolic reprogramming to make better use of resources and continue proliferating [36]. The classical response to hypoxia is activation of hypoxia inducible factor HIF that functions as a master regulator of anaerobic metabolism and neo-angiogenesis [62]. In addition to HIF response, oncogenic KRAS, other oncogenic mutations, as well as growth factors can also up-regulate glucose metabolism thereby promoting fitness in hypoxic microenvironment [39].

One of the hallmarks of cancer is the ability of cancer cells to kick start formation of new vasculature, neoangiogenesis [67]. Indeed, the cancer cells themselves can secrete angiogenic factors through inherent mechanisms or environmental mechanisms via HIF response [67]. Furthermore, factors secreted by PSCs such as osteonectin are known to induce neangiogenesis [68]. This means that the fibrotic stroma creates dynamic microvasculature leading to periodic cycles of moderate to deep hypoxia, which will favor selection of Warburg effect metabolism. Metabolic reprogramming is reminiscent of ecological studies that have shown success of invasive species through better utilization of limited resources to grow their population. A more elaborate depiction of metabolic reprograming and its role in cancer cell fitness will be provided in the upcoming sections.

The Immune System in PDAC

The immune system is one of the marvels of evolution that contributes to the overall homeostasis of multicellular organisms by eliminating foreign invaders through the combined action of specified cells, tissues, and organs. Thus, the immune system is critical in cancer prevention as it can recognize malignant cells as foreign entities and eliminate them. Evasion of immune response is critical for emergence of initial somatic mutations and metastasis, as cells need to avoid detection and continue their oncogenic processes [69]. Several lines of evidence suggest that the microenvironment of early pancreatic duct lesions is immunosuppressed [70]. Therefore, the immune system imparts selective pressure for mutant cells in early neoplastic lesions to develop mechanisms of immune evasion and produce a phenotype that can evade the immune response, as well as, other growth constraints. Where as in PDAC tumors, there is evidence the TME leads to T-cell suppression through accumulation of CD4 positive regulatory T-cells (T_{reg}), M2 tumor-associated macrophages, and myeloid-derived

suppressor cells [70]. Accumulation of T-cells in the TME demonstrates that endogenous T-cells are functional, but upon arriving to the TME they become suppressed. Mechanisms of T-cell exhaustion include expression of inhibitory effectors (PD1, LAG3, TIM3), high-antigen tumor load, and immunosuppressive TME due to hypoxia or acidosis, as well as, limited nutrient access [71].

PDAC Metastasis

Metastasis is managed clinically as late event in carcinogenesis, but from an evolutionary standpoint it is a reflection of clonal competition and fitness levels in the primary site. However, local tissue invasion itself is not a rate-limiting step in formation of metastases; this is because clonal expansion is a random process meaning that each replication cycle can yield metastatic cells, but PDAC progression seem to be a stochastic process and TME and cell population may drive metastatic selection of clones [72]. Furthermore, more than 99% of cells that enter the circulation do not survive in blood beyond 24 hours [52]. These observations suggest metastasis will be more likely with increasing tumor cell population growth; moreover, dissemination occurs from the earliest stages of pancreatic carcinogenesis meaning that PDAC tumors undergo rapid growth early in PDAC carcinogenesis. However, early carcinogenesis is an ambiguous term as PDAC goes undetected for years before diagnosis and thus its timely progression is not well described. Nevertheless, metastasis can occur on first replication cycle, but statistically it will be more favored upon establishment of primary tumor. Interestingly, no metastasis-specific genes have been found in pancreatic cancer; instead, a substantial proportion of metastatic efficiency is determined by the genetic alterations that arise during the clonal expansion phase (*KRAS, CDKN2A, TP53 and SMAD4*) [51, 52]. Thus, the genetic features of the parental clone play an instrumental role in determining the extent to which the clone will successfully adapt and survive in

foreign microenvironments. Evidence to support the notion that metastasis develops from evolution of cancer cells in the primary site comes from genomic studies of patients with metastatic lesions showing that the metastatic cell population arises from cells in the primary tumor site identified by their unique set of passenger mutations and structural rearrangements, which are genetic markers of the life history of that lineage [73]. Additionally, mathematical models factoring immune response, phenotypes, and stochastic evolution predict metastatic success least 5–10 years following development of the parental clone [51].

These observations highlight the significance of timely adaptation of PDAC cells to the TME to achieve development of metastatic lesions. The complementary features for induction of metastasis of PDAC cells acquired in TME of the primary site that promote metastasis to the liver, lungs or peritoneum are still undiscovered. Evidence of selection for metastatic phenotype is shown in an interesting recent study of orthotopic PDAC mouse models where it was shown that organ-specific metastases are enhanced upon multi-clonal implantation [74]. Perhaps this is an artifact of competition and use of resources since PDAC cells lines have been shown to belong into three metabolic subtypes that are associated with glycolysis, lipogenesis, and redox pathways [75]. This implies that metabolism of PDAC cells plays a critical role in the phenotype of PDAC cells and whether they are primary site cells, metastatic, or therapy resistant. In the following sections the significance of metabolism in carcinogenesis with specific focus on PDAC.

Cellular Metabolism

One of the hallmarks of cancer is up regulated metabolism this occurs because cancer cells are unresponsive to tissue-mediated growth inhibition, as well as, loss of intrinsic growth regulators (oncogenic mutations); thus cancer cells begin to proliferate uncontrollably. In the following section the role metabolism plays in cellular energetics and growth will be addressed.

Metabolism refers to the biochemical processes within an organism through which energy and biomass are produced to sustain homeostasis and growth of an organism. Inside of a cell, nutrients are broken down and linked in a series of enzyme-catalyzed chemical reactions also known as metabolic pathways. The reactants, products, and intermediates of these reactions are also referred to as metabolites. In a metabolic pathway the product of a reaction is the substrate for the next. Critical for metabolic enzyme-driven reactions are co-factors derived from vitamins or dietary minerals. There are two types of metabolic pathways: catabolic and anabolic. The function of the former is to break down metabolites, which release energy that is harnessed by the cell while the latter utilizes energy for biosynthesis of macromolecules. These two processes compliment each other because catabolism produces energy and anabolism consumes it during biosynthetic reactions. **Figure 8**, highlights catabolic-anabolic relationship.

Glycolysis, breakdown of glucose, is a ubiquitous metabolic pathway in all living cells. The glycolytic pathway takes place in the cytoplasm producing 2 pyruvate molecules and 4 adenosine triphosphate (ATP) molecules through substrate level phosphorylation of ADP (ADP→ATP). ATP's phosphate bonds hold potential energy, which generate free energy used by the cell when they are broken—hence ATP is the energy currency of the cell. However, 2 reactions in glycolysis require modification through phosphorylation, hexokinase [glucose→glucose-6-phosphate] and

phosphofructokinase [fructose-6-phosphate→fructose-1,6-bisphosphate], because of this expenditure of energy glycolysis only produces a net of 2 ATP molecules for the cell.

Approximately, 2.3 billion years ago the atmospheric levels of O_2 increased to 35%, today they are 21%, this caused a branching point in metabolism [76]. Glycolytic process uncoupled from oxygen became known as anaerobic metabolism or aerobic metabolism when coupled to oxygen. In anaerobic metabolism glucose-derived pyruvate is converted to lactic acid through a process called lactic acid fermentation. This process also reduces glycolysis co-factor nicotinamide adenine dinucleotide, NAD^+ →NADH, which can then be recycled for sustained glycolysis. In aerobic glycolysis, glucose-derived pyruvate is delivered to the mitochondrion—the cell's 'engine room,' where it is converted into acetyl-coA via pyruvate dehydrogenase complex. Acetyl-coA is oxidized in a series of reactions collectively known as the tri-carboxylic acid (TCA) cycle using co-factors nicotinamide adenine dinucleotide (NAD^+) and flavin adenine dinucleotide (FAD^+), which are reduced in the process—NADH, $FADH_2$. Energy is generated through the electron transport chain (ETC), a system composed by a series of protein-complexes installed in the inner membrane of the mitochondria. The ETC conducts a series of reduction/oxidation reactions moving electrons from NADH and $FADH_2$ to O_2, which picks up protons and converts to water. Complex I,II, III and IV of ETC move protons produced through oxidation reactions in TCA cycle, into the inter-membrane of the mitochondria generating an electrochemical gradient. While, Complex V of the ETC re-cycles protons back into the inner membrane of ETC using the potential energy movement of protons down their concentration gradient for ADP→ATP phosphorylation. This whole process is possible because of oxygen is the final electron acceptor—hence the term oxidative phosphorylation. Reference [77].

Metabolic Control

Aerobic metabolism generates more energy allowing approximately 1000 more reactions to take place compared to anaerobic metabolism, which led to generation of new metabolites enabling cellular biosynthetic potential [76]. For example, the oxidative decarboxylation of pyruvate to acetyl-CoA commits the carbon atoms of glucose to two principal fates: oxidation to CO_2 by the citric acid cycle or incorporation into lipid via fatty acid synthesis. In eukaryotic cells the major metabolic control is through compartmentalization with glycolysis occurring in the cytosol and oxidative metabolism in the mitochondria. Enzymes are potential sites for regulation of metabolic pathways, more specifically enzymes catalyzing irreversible reactions. The significance of metabolic regulation is crucial for maintaining basal metabolic rates in normal cells.

In glycolysis, the reactions catalyzed by hexokinase, phosphofructokinase, and pyruvate kinase are irreversible; thus, each of them serves as a control site with their respective function regulated by the reversible binding of allosteric effectors or by covalent modification. Allosteric regulation is mediated by a feedback loop of reaction and pathway products. In addition, the expression of these enzymes is tightly regulated by transcription factors in response to metabolic needs. The oxidative decarboxylation of pyruvate to acetyl-CoA is a critical branch point in metabolism. For this reason the activity of the pyruvate dehydrogenase complex is controlled by several means such as negative feedback of reaction products, as well as, covalent modification through Phosphorylation of the pyruvate dehydrogenase complex by pyruvate dehydrogenase kinase; while deactivation is reversed by pyruvate dehydrogenase phosphatase. Increasing the $NADH/NAD^+$, acetyl CoA/CoA, or ATP/ADP ratio promotes phosphorylation and, hence, deactivation of the complex. For this reason the pyruvate dehydrogenase complex is switched off when the energy level is high and biosynthetic metabolites are abundant. The rate of the citric acid cycle is precisely adjusted to meet

an animal cell's needs for ATP. The primary control points are isocitrate dehydrogenase and α-ketoglutarate dehydrogenase enzymes through allosteric inhibition of pathway products. Thus, regulation of TCA cycle is primarily due to NADH/NAD+ or ATP/ADP ratio. Reference [77].

Metabolic regulation a balance for growth and survival

Consider the fact that the biomass of microbes nearly doubles that of higher multicellular organisms. Needless to say, the number of microbial species far outnumbers that of multicellular organisms. This is because, microbes have been selected to grow as fast as possible when nutrients are available. Physiologically, this means that they need to re-wire their metabolic biochemistry from ‘survival mode’ to ‘growth/replication mode’ in order to achieve this there must be a switch from basal metabolic rate to a metabolic rate competent to generate biomass required for exponential growth. Upon malignant transformation, cancer cells gain fitness and adopt a metabolic phenotype that is reminiscent of microbial exponential growth in order to produce an exact copy of itself.

As previously described, normal cell function is maintained through the catabolic metabolism of glucose to generate free energy through oxidative phosphorylation. To sustain homeostasis and remain functional normal cells also need to undergo anabolic metabolic processes, which requires harnessing of biomass from nutrients to synthesize macromolecules as needed [56, 78]. ATP may be used for activation of some reactions, but anabolic processes need biomass and cofactors. Thus, it is illogical that all carbons would be maximized for energy production. For example, the synthesis of palmitate, the most common saturated fatty acid found in plasma membrane of animals, requires 7 molecules of ATP, 8 molecules of acetyl-CoA, and 28 electrons from oxidized $NADP^+$,

NADPH→ $NADP^+$. Similarly, the synthesis of amino acids and nucleotides requires more biomass and NADPH than ATP molecules. Oxidative metabolism of glucose yields a maximum of 36 ATP molecules and 2 NADPH molecules. NADPH will only be produced if glycolysis is branched into the oxidative arm of the pentose phosphate pathway (PPP). Thus, the amount of ATP produced through oxidative metabolism of glucose far exceeds its need. In the example of palmitate, a glucose molecule may provide 6 carbons while 16 carbons are required for the fatty acyl chain. Similarly, a single glucose molecule can only reduce 2 $NADP^+$ units; therefore, 7 glucose molecules are needed to make palmitate. These examples illustrate the fact that glucose cannot be committed to ATP synthesis entirely as it is counter productive for anabolic processes. Reference [56, 79]

In addition to glucose, the amino acid glutamine is essential for bioenergetics, redox, and biosynthetic reactions. This is because glutamine is the most abundant amino acid in blood and muscle, for this reason, glutamine has become an essential metabolite for cell functions. For example, glutamine can be broken down through glutaminolysis to derive acetyl-CoA and generate energy via oxidative phosphorylation. Glutamine can also undergo anabolic pathways, annaplerosis, to synthesize macromolecule such as, non-essential amino acids (NEAAs), purines, pyrimidines, and fatty acids. Furthermore, glutamine is used to make antioxidant molecule glutathione. For this reason many cells shift their metabolism to be more dependent on glutamine for their survival, growth, and proliferation. Cells that become glutamine dependent show that up to 90% of their oxaloacetate is derived from glutamine. Glutaminolysis is a process exclusive to the mitochondria, it begins with uptake of glutamine and its conversion to glutamate by glutaminase enzymes (GLS1/GLS2), and glutamate is then converted into α-ketoglutarate via two diverging pathways. The canonical anaplerotic pathway occurs through action of glutamate dehydrogenase (GLUD). The second

pathway is via the non-canonical anaplerotic pathway is conducted by glutamate transaminase enzymes (GOT). Metabolic flux through the canonical pathway generates ammonia and NADH from decarboxylation of α-ketoglutarate in the TCA cycle. An interesting fact is that ammonia is an inducer of autophagy, a system through which glutamine is provided for metabolism. Contrastingly, the non-canonical pathway generates NEAAs instead of ammonia, including aspartate, alanine, and phosphoserine. Glutamine-derived OAA can condense with acetyl-CoA to make citrate, which is a substrate for ATP-citrate lyase (ACLY) for de novo lipogenesis via fatty-acid synthase (FASN). Glutamine-derived fatty acids can also be made through cytosolic isocitrate dehydrogenase (IDH1) in a caboxylation reaction. Similarly, glutamine-derived metabolites can be shuttled out of the mitochondria through shuttling mechanisms. For example, glutamine-derived aspartate can take part on the malate-aspartate shuttle and produce NADPH via malic enzyme 1 (ME1) or NADH through ME2 in the mitochondria compartment. Aspartate can also be converted to asparagine and participate in nucleotide synthesis pathways. Glutamine has been shown to play a critical role in cell metabolism and cells that rely solely on glutamine die upon depletion unless they undergo adaptation to glutamine depravation. Indeed, cells that are glutamine independent have increased expression of pyruvate carboxylase (PC), which converts pyruvate to OAA that is used to maintain TCA cycle if PDH is inhibited. Thus, when cells are stimulated to undergo growth the biosynthetic components: energy, biomass, and co-factors of reactions can be generated from glucose, amino acids (glutamine), and lipids (fatty acids). Reference [80]. Figure 9 highlights the canonical metabolic pathways.

Cancer Cell Metabolism

A hallmark of cancer is the metabolic shift characterized by a marked increase in glucose uptake and subsequent lactic acid fermentation, compared to normal cells, in the presence of oxygen. German scientist Otto Warburg, hence the name Warburg effect discovered this phenomenon a century ago [81]. At the time, Warburg's observation contradicted the Pasteur effect dogma, which described a metabolic shift between lactic acid fermentation and oxidative metabolism depending on oxygen availability. For nearly half a century, it was widely believed that cancer cells lacked functional mitochondria. However, the discovery of oncogenes and tumor suppressors, as well as, the improvement in genomic studies removed interest from cancer metabolism studies until the beginning of the 21st century. In the previous section, I have explained metabolic regulation and the theoretical role of anabolic metabolism upon growth stimulation. Cancer is characterized by uncontrolled cellular growth which, means that to produce viable cells during mitosis, a cancer cell must double its cellular content, which implies the synthesis of billions of nucleotides, lipids, and amino acids [56, 78]. Thus, cancer cells hijack cellular metabolism to maximize biosynthetic potential to produce macromolecules, energy, and maintain redox homeostasis. To achieve this functions and increase proliferation cancer cells exponentially increase glucose and glutamine uptake. Indeed, C_{13} glucose/glutamine uptake in glioblastoma shows that up to 90% of glucose and 60% of glutamine is committed to lactate synthesis [82]. The high rate of conversion of glucose and glutamine into lactate seen in cancer cells is mediated by lactate dehydrogenase A (LDHA). Inhibition of LDHA has shown to diminish growth of cancer cells possibly as it will limit secretion of excess carbon in a process recycles NADH, which is needed for continuous use glycolysis and TCA cycle metabolism [83].

Similarly, increased glucose/glutamine uptake allows cells to generate NADPH necessary for anabolic pathways and redox homeostasis.

Cells in a multicellular organism do not experience shortage or resources; thus, there is no selective pressure for optimizing metabolism for maximum ATP yield. However, selection for optimization of anabolic metabolic response does take place in effector cells of the immune system. This is because upon damaging injuries the body has to heal quickly in order to survive. Thus, it's only logical that a fast responding immune system evolved. Cells capable of utilizing glucose and glutamine more effectively will proliferate faster. In the next section, I will elaborate on the development of PDAC metabolism.

Hypoxia: Orchestrator of PDAC glycolytic metabolism

Increased expression of key glycolysis enzymes in PDAC leads to a robust increase in metabolic activity and Warburg effect metabolic phenotype. These enzymes include, hexokinase 2 (HK2), phosphoglycerokinase 1 (PGK1), pyruvate dehydrogenase kinase isozyme 1 (PDK1), lactate dehydrogenase A (LDHA), enolase 2 (ENO2), and pyruvate kinase muscle 1 and 2 (PKM1,2), as well as, increase espression of membrane transporters such as glucose transporter 1 (GLUT1) and monocarboxylate transporters 1 and 4 (MCT1,4) [66]. This metabolic shift is likely due in large part to stabilization of HIF complex because of hypoxia induced by strong desmoplastic reaction. HIF is regulated by oxygen perfusion therefore; when oxygen is depleted the HIF1α subunit of the complex is stabilized allowing for its interaction with HIF1β and subsequently recruitment to the nucleus as a complex to begin transcription of HIF response elements (HRE) [62, 84]. The HIF response is a ubiquitous response for oxygen depletion and survival is promoted by increase in glycolysis and induction of neo angiogenesis [62, 84]. HIF1α

has an oxygen dependent degradation domain that is hydroxylated by propyl hydrolylases (PHDs). Hydroxylation of HIF1α allows for recognition by the von-Hippel Lindau binding protein, which is an E3 ubiquitin ligase that signals for HIF1α degradation [62, 84]. Because activation of HIF complex is a ubiquitous response mutations in the pathway are uncommon; however, the renal-cell carcinoma cell line RCC4 has shown mutations to VHL and as a result there is increased expression of HIF1α [85]. The role of HIF response in PDAC is undisputed and nearly 90% of tumors have increased expression of HIF1α. As a result PDAC cancers have increased glycolysis followed by high rate of lactate secretion. **Figure 10** highlights HIF1α stability and HIF complex metabolic response.

However, it is not conclusive to say that stabilization of HIF1α induced by the hypoxic TME is the cause of Warburg effect in PDAC. For example, hyperglycemia occurs as a result of diabetes type II, which predisposes for PDAC and it's often a symptom of PDAC as well; replication of hyperglycemia conditions in vitro indicates an increase in HIF1α expression in normal oxygen conditions [86, 87]. Stabilization of HIF1α has also been demonstrated to occur upon growth factor signaling, PI3K, heat-shock protein 90 (HSP90), and cyclooxygenase-2 activity (COX2) [88-91]. Other factors increasing HIF1α protein expression include ROS stress, nutrient depravation, growth factor signaling, metabolic disturbances such as increases succinate, as well as, protein-protein interactions resulting in enhanced stability, acetylation, or phosphorylation [84, 92]. For example, a study from our lab showed that the cytoplasmic terminal of MUC1 associates with HIF1α promoting its stability and subsequent engagement in transcription of glycolytic genes [93, 94]. However, it is very likely that constitutive stimulation of HIF in PDAC is because of cyclic hypoxia throughout premalignant lesions and during establishment of primary tumor because of dynamic fibrotic stroma [95]. This

is consistent with the somatic evolution of PDAC to adopt Warburg effect metabolism independent of HIF, as it occurs *in vitro*. Thus, hypoxia response is critical because cells need to adopt this metabolic behavior to survive inconsistent oxygen perfusion due to growing stroma; where as normal ductal cells cannot survive prolonged absence of appropriate oxygen levels because hypoxic metabolic response will lead to tissue damage and turnover due to disruption of pH dynamics in exocrine pancreas [29]. Furthermore, PDAC cell lines with HIF1α depletion still exhibit increased glycolytic metabolism clearly indicating that the metabolic phenotype is maintained through oncogenic transformation, as it would promote fitness over normal cells [96].

KRAS Orchestrator of PDAC glycolytic metabolism

Oncogenic KRAS occurs almost ubiquitously in PDAC found in low-grade PanINs and with magnified expression in PDAC cells [38]. Its constitutive activity triggers several signal transduction cascades such as PI3K-AKT-mTOR, RAF-MEK1/2-ERK1/2 (MAPK), Ral-GEF, and Rho-Rac, which eventually relays signal to activate transcription mediators in the nucleus including c-MYC, NFκB, E2F, HIF1a, AP-1, and C-jun, Introduction Figure 4 [97, 98]. In this way, oncogenic KRAS effectively regulates a plethora of cellular activities such as cell growth, survival, migration, and metabolism. For these reasons oncogenic KRAS is essential for PDAC carcinogenesis. However, its expression alone is not sufficient to induce PDAC. As demonstrated in mouse models bearing only oncogenic KRAS that gives proliferation to cells in PanIN, but does not progress to PDAC likely due to tissue growth constraints [99]. Several lines of evidence suggest KRAS selects for Warburg effect phenotype. For example, transfection of oncogenic KRAS into human pancreatic ductal cells causes a moderate metabolic increase compared to PDAC cells; similarly, oncogenic KRAS (G12V) in embryonic kidney cells is known to induce up regulation of glycolysis at the cost of mitochondrial

dysfunction [100]. These examples also allude to the somatic evolution of PDAC to adopt Warburg effect metabolism.

Oncogenic KRAS takes on metabolism through several effector pathways of which the most prominent are the PI3K-Akt and MAPK pathways [98, 101]. The PI3-Akt signaling pathway increases the transcription and translation of GLUT1, increase activity of phosphofructo kinase (PFK), localization of HK2 to the mitochondria thereby increasing rate of the reaction of glucose→glucose-6-phosphate, and stabilization of HIF1α [102]. While the activation of MAPK pathway increases transcription rate of HIF1α [103]. Increased expression of HIF1α through RAS activity has been demonstrated in other RAS driven cancers through inhibition of PI3K and ERK [102-104]. Similarly, wild-type RAS molecules overexpressed in in a variety of cancer types can be targeted by inhibition of farnesyl transferase, which anchors RAS molecules to the plasma membrane, resulting in metabolic stress due to reduced HIF1α expression [105-107].

1. KRAS driven glutamine addiction

As previously stated, glutamine is the most abundant metabolite in blood and, along with glucose, it's the major substrate for metabolic reactions in PDAC. Glutamine in PDAC is preferentially metabolized via the non-canonical anapleurotic pathway because of oncogenic KRAS expression. Indeed, a study by Son et al. found that KRAS plays a direct role in glutamine metabolism by increasing expression of GOT1 and reducing GLUD1 expression. In this way, oncogenic KRAS guides metabolic flux through the non-canonical pathway. These findings show that PDAC up regulates non-canonical glutamine metabolism in order to increase NADPH levels in order to maintain non-cytotoxic ROS levels. Furthermore, this observation makes up for PDAC preference for non-oxidative PPP by providing the NADPH that otherwise would be generated through

oxidative PPP. Reference: [108]. **Figure 11** highlights KRAS driven non-canonical anaplerotic glutamine metabolic pathway.

2. KRAS re-directs glucose to PPP

The Pentose phosphate pathway (PPP) is a biphasic, oxidative and non-oxidative, cytosolic process that utilizes glucose, as the initial metabolite, and glycolytic enzymes to generate initial metabolic molecules. However, unlike glycolysis PPP is an anabolic process that produces 5 carbon sugars. The oxidative branch of PPP takes glcose-6-phosphate from hexokinase reaction as its initial metabolite, which is then converted to 6-phosphogluconolactone via glucose-6-phosphate dehydrogenase (G6PD) [glcose-6-phosph→6-phosphogluconolactone]. This irreversible reaction commits glucose to oxidative PPP and reduces 2 NADP→2 NADPH. Subsequently the reaction 6-phosphogluconolactone→6-phosphogluconate takes place via hydrolase gluconolactonase. The last step in oxidative PPP is the decarboxylation reaction of the 6-carbon molecule via 6-phosphogluconate that reduces 2 NADP→2 NADPH [6-phosphogluconate→Ribulose-5-phosphate]. Production of NADPH through oxidative PPP is essential for suppression of oxidants using glutathione. This is because glutathione reductase (GR) oxidizes NDPH and reduces glutathione (GSSG+NADPH→GSH+$NADP^+$), which is then oxidized by glutathione peroxidase (GPX) moving the electrons to hydrogen peroxide converting it to water [GSH + H_2O_2→GSSG + $2H_2O$]. This is noteworthy because GR is highly expressed in PDAC, as well as, high levels of NADPH indicating the significance of ROS homeostasis. Reference: [109, 110].

The non-oxidative PPP uses the 5-carbon sugar to generate intermediates for synthesis of nucleic acids and nucleotides. If there is a shift in metabolic demand for

ATP, pentose sugars from non-oxidative PPP can be recycled as intermediates of glycolysis, recycled as fructose-6-phosphate and glyceraldehyde-3-phosphate. Similarly, glycolysis intermediates can be used for nucleotide synthesis. This is the case of PDAC where oncogenic KRAS simulates glucose uptake and conveys glycolysis intermediates for biosynthesis of nucleotides and nucleic acids. The exact mechanism is not well understood; however, inhibition of MAPK and ablation of c-MYC results in decreased metabolic flux to non-oxidative PPP. Oncogenic KRAS makes up for loss of NADPH synthesis through oxidative PPP by induction of non-canonical glutamine metabolism, which increases NADPH needed for ROS homeostasis. Reference: [96, 109]. **Figure 11** highlights KRAS driven non-oxidative PPP pathway.

3. Oncogenic KRAS Redirects Glucose to HBP

The hexosamine biosynthetic pathway (HBP) links glucose metabolism and glutamine metabolism through Glucosamine-fructose-6-phosphate aminotransferase (GFPT) [fructose-6-phosphate+glutamine→glucosamine-6-phosphate]. This is the initial precursor in HBP for synthesis of UDP-GlcNAc (uridine diphosphate n-acetylglucosamine), which is used by O-GlcNAc-transferase (OGT) for protein O-GlcNAc glycosylaton. Interestingly, PDAC have high levels of O-GlcNAc glycosylated proteins because of up regulation of HBP pathway enzymes and OGT, as well as, low levels of O-GlcNAcase (OGA), which catalyzes de-glycosylation. KRAS increases glucose and glutamine uptake, as well as, the expression of HBP rate limiting enzyme GFPT. Increased HBP activity has been linked with tumor invasion and metastasis. Reference: [96, 111]

Post-translational modification of PFK1 via O-GlcNAc glycosylation has been shown to inhibit its activity thereby fluxing G6P to oxidative PPP or HBP [112].

Furthermore, O-GlcNAc glycosylation stabilizes important transcription factors such as p53, c-MYC, and β-Catenin [113]. Increased metabolic flux through HBP promotes post-translational and epigenetic alterations that promote carcinogenesis by increasing aneuploidy, as well as, secretion of insulin, TGFβ, and FGF [113, 114]. Thereby, promoting mutability and communication to other cancer cells and tumor stroma resident cells such as PSCs. Interestingly, HBP also modulates tyrosine kinase receptor signaling in PDAC shown by inhibition of OGT, which caused decrease in EGFR, ErbB2, ErbB3, and IGFR [113, 114]. Logically, glucose starvation reduces metabolic flux to HBP thereby decreasing protein glycosylation, but it must be highlighted that this leads to apoptosis by unfolded-protein-response [115]. Thus, protein glycosylation is critical for protein stability and function, which will be essential for PDAC cell proliferation. Furthermore, protein glycosylation is likely to mediate cellular differentiation by modulating cell-cell adhesion, responsiveness to GFs, immune system evasion, and signal transduction, through mechanisms not yet known. Thereby, HBP metabolic flux may play a critical role in neoplastic lesions. **Figure 11** highlights KRAS driven HBP pathway.

4. Role of KRAS in fatty acid metabolism

In lung adenocarcinoma, it has been demonstrated a direct role of KRAS in fatty acid synthesis through activation of ERK2 pathway that increases FASN expression [116]. However, in PDAC the exact mechanisms of FA metabolism are not fully elucidated, but PDAC cell lines show increased expression of FAO and FASN. Studies comparing growth kinetics of normal ductal cells versus PDAC cells showed that a high lipid diet enhances PDAC cells more [117]. This indicates that PDAC cells can be stimulated to uptake lipids and used them for energy and biomass generation i.e. membrane backbone. These observations have been recapitulated using PDAC mouse

models fed a high fat diet versus low fat diet [118]. The high fat diet a marked increase in metabolic rate and energy production thanks to an increase in molecules that regulate uptake and oxidation of FAs. Indeed, high fat diets are known to be factors increasing risk of PDAC [119]. The role of FA and its regulating mechanisms in PDAC are not well known. Interestingly, it has been found that omega-3 FAs cause cancer cell death while omega-6 FAs correlate with incidence of cancer, including PDAC [119]. This may be because omega-6 FAs increase obesity, which incidentally increases cancer risk.

Role of p53 in Metabolic Regulation

Tumor suppressor p53 has long been shown to play key role in response to stress such as DNA damage, hypoxia, and oncogenic activation. Upon these events, p53 stalls growth (senescence) and induces apoptosis if damage is irreversible. To achieve these functions p53 must regulate the metabolic response to sustain cells viable upon stress. To intervene in metabolism, p53 interjects at many points in both glycolysis and oxidative phosphorylation and works to balance these metabolic pathways thereby inhibiting Warburg effect and growth associated with growth and carcinogenesis [120]. Therefore, loss of function of p53 will result not just in induction of mutations due to failing DNA damage response, but also loss of metabolic regulation furthering PDAC growth.

p53 increases the expression of cytochrome C oxidase 2 (SCO2) a critical mediator of oxidative phosphorylation, as well as, GLS2 influencing canonical glutamine metabolism to sustain TCA cycle and oxidative metabolism [121, 122]. p53 modulates glycolysis as well by increasing expression of transporters GLUT1,4, and TIGAR (*TP53*-induced glycolysis and apoptosis regulator) whose metabolic role is through inhibition of fructose-6-phosphate conversion to fructose-1,6-bisphosphate by increasing activity of

frucosebisphosphotase 2, which carries out the reverse reaction [120, 123]. In this way, p53 would redirect glycolysis intermediates to the oxidative PPP for production of NADPH and nucleotides. This function is critical for survival of cells upon replication and reactive oxygen (ROS) stress; p53 also reduces glycolysis by decreasing expression of phosphoglycerate mutase (PGM) [120]. In this light, it is easier to contextualize why deregulation of p53 is so critical for carcinogenesis. Furthermore, because of the contrasting role of p53 in inducing glycolysis for ROS or DNA damage response and induction of oxidative metabolism to suppress high glycolytic rate, the stability of p53 is critical to maintain basal metabolism and overall homeostasis. In PDAC the *TP53* mutation affects the p53 DNA binding domain thereby affecting transcription only the genes with a p53 response element; while other protein-protein interactions would remain thereby allowing formation transcription complexes where p53 is an activator of transcription [123]. For example, it has been observed that cells with p53 mutations to its DNA binding domain do not affect p53-related increased expression of glucose transporters [124]. Similarly, mutant p53 in liver tumors is implicated in high expression of HK2 thereby increasing the glycolytic rate of cancer cells [125]. Furthermore, the difference between basal levels of p53 versus increased levels of p53 during severe or sustained stress might allow for the transcription of different sets of genes and a different metabolic response. Thus, in PDAC p53 transcription may still occur as cellular stress will induce its expression. **Figure 12** highlights p53 metabolic regulation.

Increased expression of p53 has been demonstrated to increase intracellular ATP levels by up regulating oxidative phosphorylation as demonstrated in human colon cancer cell line HCT116 with high p53 versus HCT116 cells depleted of p53 [126]. This is achieved by increased expression of SCO2 a major component of the cytochrome c oxidase complex in the ETC, complex IV. p53 also induces the expression of GLS2

which converts glutamine to glutamate which can be used in the TCA cycle-ETC pathway when metabolized to α-ketoglutarate to generate energy or glutamate could be used in the synthesis of GSH to fight ROS stress [122]. Therefore, by increasing ATP yield p53 down regulates glycolysis through allosteric inhibition of its enzymes, previously reviewed. Wild-type p53 induces the transcription of p53-modulator-of-apoptosis (PUMA) and BCL2-associated X protein (BAX) [127]. These proteins work together to create a pore in the outer membrane of the mitochondria and cause release cytochrome-c, which is used to activate caspase-mediated apoptosis. There is an interesting link between p53 and glycolysis, as it has been shown that increase glucose uptake suppresses PUMA expression [128]. Furthermore, pAKT causes inactivation of PUMA thereby enhancing glycolysis [129]. The role of p53 in regulation of cellular metabolism is highly complex because of the plethora of cell functions associated with it. PDAC p53 mutations targets its DNA binding ability while other protein-protein activity is not altered, which will have an effect in p53 functions such as metabolic regulation. Because of the prevalence of Warburg effect metabolism it is likely that mutant p53 plays a role in maintaining glycolysis it as evidenced by increased PPP metabolic flux in PDAC or it may be that its metabolic regulating function is inhibited.

1. p53 regulates FA metabolism

FAs are important catabolic metabolic intermediates because they can undergo beta-oxidation to generate acetyl-CoA, which produces energy through TCA-ETC pathway [79]. Upon stress from DNA damage and glucose deprivation, p53 increases the expression of guanidinoacetate N-methyltransferase (GAMT), which mediates the conversion of glycine-derived guanidinoacetate to creatine for substrate level phosphorylation of ADP thereby increasing ATP/ADP ratio, as it occurs in glycolysis. Cancer cells can alter their metabolism by increasing de novo fatty acid (FA) synthesis

irrespectively of the levels of extracellular lipids [130]. Similarly, increased expression of FAO occurs in the livers of wild-type p53 mice, suggesting that p53 plays a role in energy maintenance through the FAO pathway [131] and FASN is a conserved p53 target [132]. These observations suggest that wild-type p53 plays a role in linking FA metabolism to FA synthesis while mutant p53 leads FA metabolism to oxidative pathways. The role of mutant p53 in PDAC metabolic regulation has not been elucidated.

Tumor Microenvironment Acidification

Water is universal solvent of life, with a high tendency to undergo self-ionization $[H_20] \leftrightarrow [OH^-]+[H^+]$. However, free protons do not exist in solution so the equation is fixed to $[H_20] \leftrightarrow [OH^-] +[H3O^+]$. The rate at which water ionizes (K_w) is defined by the equation $K_w=[OH^-][H^+]/[H_20]$, which has been determined to be equal to 1.00 x 10^{14} mol^2 dm^{-6} at 25°C and 2.4 10^{14} mol^2 dm^{-6} at 37°C. Therefore, the potential concentration of hydrogen ion $[H^+]$, pH, or hydroxide ion $[OH^-]$, pOH, at equilibrium is defined by $[OH^-]x10^7+[H^+]x10^7=[H_2O]10^{-14}$. The pH and pOH can then be calculated by taking the negative logarithm (-log) of the concentration ($_{-log}$[H+] or $_{-log}$[OH-] which means that at equilibrium 7+7=14 since $pH+pOH=K_w$. However, because the body has an internal temperature of 37°C and K_w=2.4 10^{14} mol^2 dm^{-6}, then the pH of water at body temperature is calculated to be 6.8. The pH scale defines the concentration of H^+ in water solutions on a scale 1-14 with values <7 acidic and >7 basic. All biological solutions have a certain concentration of protons from the balance between protonation and deprotonation of water, weak acids, as well as, weak bases. For all living organisms pH regulation is critical as it regulates all biochemical functions. Changes in pH alter 3-dimential protein structures altering their function and inducing denaturation. Therefore,

living organisms must maintain a narrow pH range through acid-base reactions, acids donate protons and bases donate hydroxide ions. Acid-base reactions exchange hydrogen ions with neutral molecules such as water (Arrhenius $HA+H_2O \leftrightarrow A^- + H_30^+$) and to electrically charged ions such as ammonium, hydroxide, or carbonate (Bronsted Lowry $HA + B \leftrightarrow A^- + HB$).

The equilibrium between protons and unpronotated molecules can be described by the acid dissociation equation, K_a, where $K_a=[H^+][A^-]/HA]$. Complex solutes such as biological molecules i.e. proteins are often given several K_a values because of their different proton binding sites. For this reason, the status of the protonatable site will vary with respect to pH. For example, at low pH due to high $[H^+]$ all protonatable sites will be filled, but with decreasing pH, low $[H^+]$, not all proton sites will be filled. Furthermore, because any given molecule has multiple K_a it can be titrated by adding base equivalents. Titration can be described by $pH=pK_a-_{log}([HA]/[A^-])$ thus if the pK_a is equal to the pH the concentration of base and acid are equal. Thus, a molecule with multiple protonatable sites also has multiple pK_a values and isolectric points, $pI= (pK_a1-pK_a2)/2$, useful to indicate the pronation status of a molecule at a measured pH. Molecules with multiple protonatable sites are of major biological significance because they can function as buffers useful in preventing major pH changes that would alter cell function. Indeed, the sensitivity of proteins to pH changes is exceptional as it is a form of post-translational modification therefore ionization of a key amino acid will alter protein function. For this reasons, it is not surprising that only a narrow range of pH is permissible for livelihood of eukaryotic cells. Reference:[109, 133]

Because of cellular metabolism, cells produce CO_2 and lactic acids as a result of metabolic activity cells are net acid producers and the pH_i has a tendency to fall. This is because lactic acid has a low pK_a and deprotonates producing $lactate^-$ and H^+ in

physiological pH, which is slightly basic ~pH 7.4. Meanwhile, accumulation of CO_2 will promote its hydration to produce carbonic acid, which also has a low pK_a relative to pH_i and will deprotonate to produce bicarbonate and release of a proton. Therefore, when cells increased their metabolic rate they will face a dangerous challenge to maintain pH_i because buffers can only reduces amplitude of pH changes but cannot remove excess protons. Thus, cells have to resolve to membrane transport. It is because the ability of biological membranes to selectively allow the passage of molecules that gives rise to difference between pH_i and pH_e, through selective transport of protons, as well as, molecules that release or take up protons such as CO_2 thereby effectively maintaining pH_i. The biological significance of protons highlights the essential function of regulating pH and to adapt to pH changes. In the following sections I will describe the role of metabolism and production of metabolic acids causing a decrease in pH of the TME. Reference: [109, 133, 134].

pH of Cancer Cells

Physiological studies have established that oxygen and glucose diffusion in human tumors is approximately at a radius of 200 μm and 280 μm from blood vessels, respectively [135]. However, beyond the oxygen diffusion limit the tissue becomes necrotic. However, hypoxic areas where oxygen tension is 1% or less is associated with increase metastatic potential of cells and poor patient outcome. This is paradoxical in terms of fitness considering that nutrients will also be less and the fact that cancer cells proliferating cells require massive amounts of nutrients to generate biomass needed to generate a daughter cells, as previously described [56]. In addition to the challenge of oxygen and nutrient deprivation, cells in the PDAC TME must also endure the stress from excess metabolic acids produced by proliferating cells. Indeed, TME acidification is emerging as a hallmark of cancer [57]. Based on these observations it would be

predicted that the pH_i of cancer cells is acidic, but it has been measured to be consistently above 7.1 so by this terms it is alkaline compared to pH_e. This trans-membrane distribution, pH_i alkaline/ pH_e acidic, of protons is not seen in normal tissues that have a balance of pH_i 7.2/ pH_e 7.4 [57]. These observations instigate two questions in cancer biology. First, how do cancer cells regulate tumor pH dynamics and, second, how those pH distribution contributes to carcinogenesis?

The byproducts of metabolism in human cells are CO_2 and lactic acid. If a cell has a high concentration of CO_2 its hydration will take place generating carbonic acid whose pK_a is 6.2 so it will deprotonate, $CO_2+H_2O \rightarrow H_2CO_3+H^+$. Similarly, lactic acid produced through fermentation has a low pK_a of 3.2 and it will also dissociate, lactic acid$+H_2O \rightarrow$ lactate$^-+H_3O^+$. Therefore, all cells are net acid producers; however, in living organisms CO_2 is moved out of cells through passive transport because of its high lipid:water partition coefficient. Thus, CO_2 modes into the capillaries where it is picked up by red blood cells and removed through respiration. While, protons are charged molecules and they cannot pass the lipid bilayer thanks to low lipid: water partition coefficient. Passive transport of H^+ via monocarboxylate transporters (MCT1,4). The rate of CO_2 and H^+ venting depends on trans-membrane concentration gradients, which physiologically are maintained by coupling with capillary blood, which delivers oxygen and removes CO_2. However, tumors are poorly perfused, especially PDAC tumors, which considerably alters venting of protons and CO_2. Because of venting impediment the CO_2 and H^+ gradient will be high in the extracellular compartment thereby limiting their passive effusion. In addition to extracellular buffers (proteins), cells have developed adaptations to surpass the gradient rate-limiting step by cell surface expression of carbonic anhydrases that accelerate the otherwise slow hydration of carbon dioxide, $CO_2+H_2O \leftrightarrow H_2CO_3 \leftrightarrow H+HCO_3^-$, thereby facilitating CO_2 and H^+ venting, carbonic

anhydrase (CA) mediates the hydration/anhydration step. Thus, hydration of CO_2 requires mobilization of protons as well leading to increase in pH_i and steady acidification of pHe. However, due to its stoichiometry coupling H^+ with lactate$^-$, requires an equal number of MCT proteins forcing cells to develop other means of transport across the membrane to maintain pH_i. This is critical for cancer cells with high metabolic rate. Reference: [57, 133, 135, 136].

In a given concentration where the K_a and buffer concentration are kept constant the only way of changing the pH is by adding base or acid equivalents, pK_a=pH+pOH and 14=pH+pOH. Where as in living cells the solution is provided with protons as a result of metabolism. Thus H^+/H^+ equivalents only lowers the pH therefore cancer cells must actively transport protons out of the cell or import their chemical equivalents $HCO3^-$ or OH^- to maintain pH_i homeostasis. To balance pH_i, membrane transporters must sense pHi changes and lead to a H^+/H^+ equivalent efflux so that the pH remains constant. To achieve this cells express membrane transporters to correct pH_i disturbance such as: Na^+/H^+ (NHE), H^+ ATPase, $Na^+/HCO3^-$ (NCB) transporter, which work against $Cl^-/HCO3^-$ exchanges (anion exchager, AE), and Cl^-/OH^- exchangers. Different types of transporters are reported in various combinations in normal cells, but bicarbonate transporter appear to be ubiquitous, were as, NHE appear to be cell type specific and dependent on conditions of environment. Most of these transporters depend on electrochemical potential of the molecules being transporter except for the H^+ ATPase that hydrolyses ATP. By coupling the activity of pH regulation membrane transporters with signaling pathways cells can fine tune the metabolic response for steady state or growth/proliferation in accordance to intrinsic (normal or oncogenic) or extrinsic factors (growth factor, hormones, microenvironment). For example, NHE1 expression is enhanced upon acidosis of extracellular space during hypoxia or upon low HCO_3^- levels

in extracellular space. The signaling mechanisms inducing pH_i control are still not understood completely.

Active transport of proton equivalents is the way cancer cells prevent acidification of pH_i, despite substantial acid load to the TME. However, this mechanism has two limitations being energy expenditure and pH_e acidification. The energy commitment challenges cancer cells because they have high ATP demand for cell functions and are restricted to oxygen for oxidative metabolism. Therefore, pH_i cannot operate at full capacity as demonstrated by decrease in NHE activity when ATP levels are low. Interestingly, the pH regulation system of removing proton equivalents does not eliminate protons with base equivalents therefore net pH_e acidification occurs since tumor tissue is poorly vascularized, as in the case of PDAC. Even if it were possible to maximize metabolic capacity in combination with pH_i control system will result in considerable acid load to TME, which will affect extracellular protein function as well as damaging the feedback for passive transport via MCT and CO_2 effusion. Therefore, in a growing tumor pH dynamics are critical for survival of the cell as they are have to devote energy for pH homeostasis and growth. Reference: [57, 133, 135, 136]. **Figure 13** highlights the pH_i regulators in cancer cells and a brief description is provided in table 2.

Hypothesis

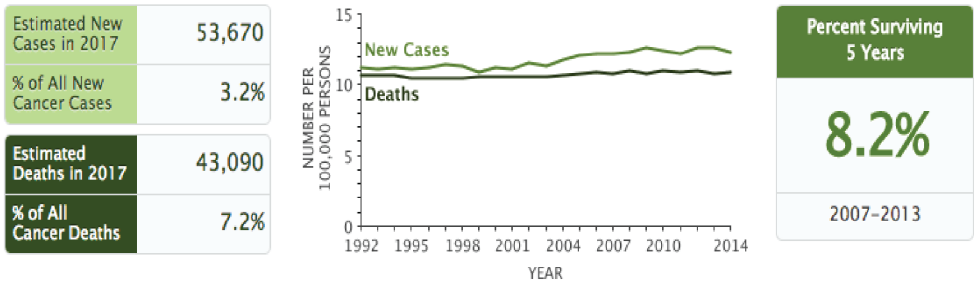
Based on these observations, the evolutionary steps of carcinogenesis include 1) acquisition of oncogenic mutation and its fixation to the tumor landscape 2) clonal expansion of mutant cells 3) increase genomic instability leading to loss of tumor suppressors i.e. *CDKN2A* or *TP53* 4) Upon loss of check point cell cycle regulation stochastic growth of tumor cells may occur, but only cells that modulate the

microenvironment by generating desmoplasia will undergo further selection to further enhance fitness of cancer phenotype over immune cells and somatic cells; thereby promoting cancer metabolic phenotype and metastasis. 5) Because of hypoxia, high IFP, and oncogenic mutations cells are driven to adopt Warburg effect metabolism. 6) Cells in hypoxic regions of the tumor continue to be selected and become the most aggressive, but their survival is limited by regulation of pH dynamics, alkaline pH_i and acidic pH_e.

In the last stage of this proposed model of the somatic evolution of PDAC, two factors fuel further growth and selection: metabolic phenotype and pH dynamics. PDAC cells are selected to grow uncontrollably, but the fibrotic TME prevents efficient ventilation of protons, which generates a challenge for regulation of pH dynamics. PDAC cells are studied in pH-buffered conditions and little information is known about the metabolic phenotype of cancer cells in low pH stress. Chapter 2 of this dissertation describes the study of PDAC cells in chronic low pH stress. For these studies, I hypothesized that in order to survive low pH stress the highly glycolytic PDAC cells must modulate their metabolism to reduce proton load into the extracellular space. I predicted a metabolic shift from Warburg effect metabolism and up-regulation of oxidative metabolism resulting in reduction of TME proton load by PDAC cells experiencing chronic low pH stress. These studies delve into the understudied low pH conditions of the PDAC TME and its effects in cellular homeostasis. These studies are highly significant to understand PDAC low pH stress response and identify therapeutic targets. **Figure 14** shows a picture representation of this hypothesis.

Chapter1 Figures

1A



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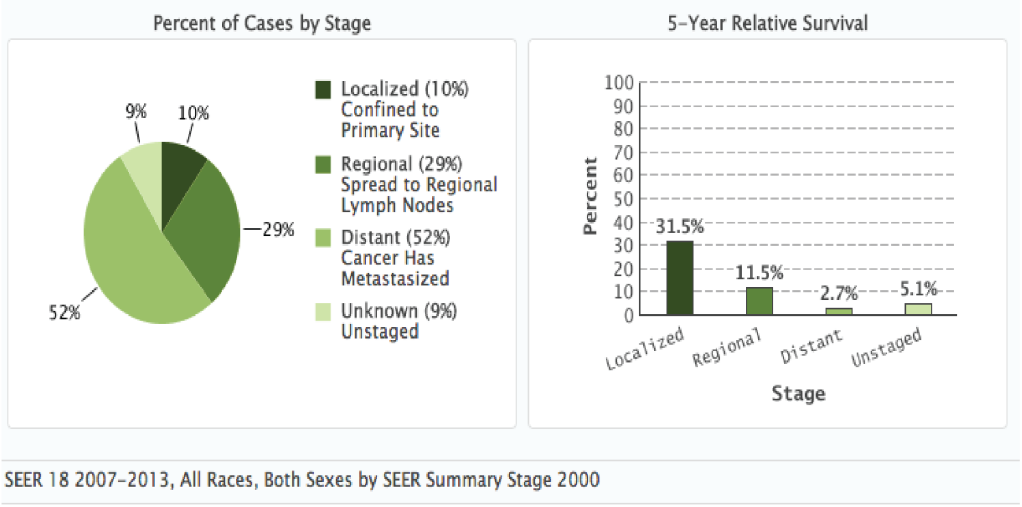

Figure 1. Statistical data of PDAC diagnoses and survival rate. 1A) The ratio of diagnose/survival ratio of PDAC and how it has remained unchanged over the past two decades. 1B) The majority of PDAC cases are diagnosed as advanced disease, which is associated with increased mortality. Seer database: https://seer.cancer.gov/statfacts/html/pancreas.html [2].

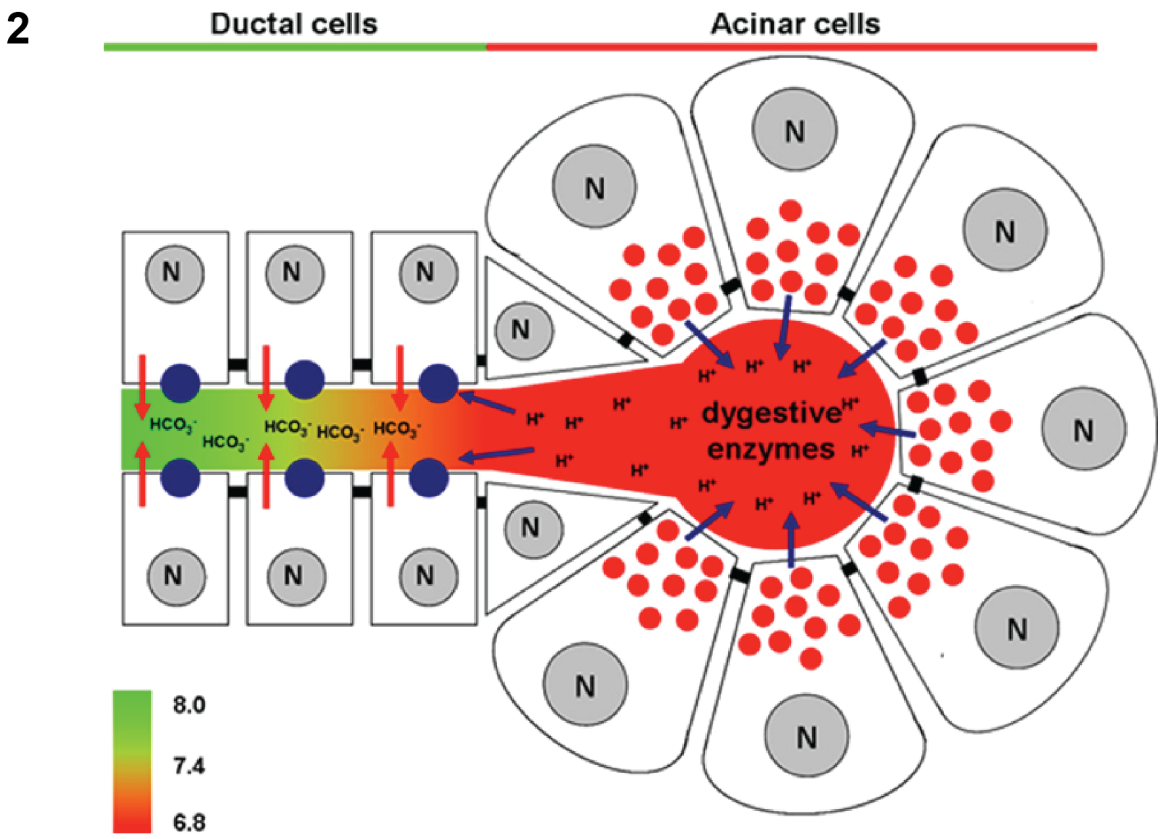

Figure 2. Changes of luminal pH in the exocrine pancreas during secretion. In physiological conditions acinar cells secrete digestive enzymes and protons, which acidify the acinar lumen. Ductal cells buffer the pH change by secreting bicarbonate. Adapted from Hegyi et al. [28]

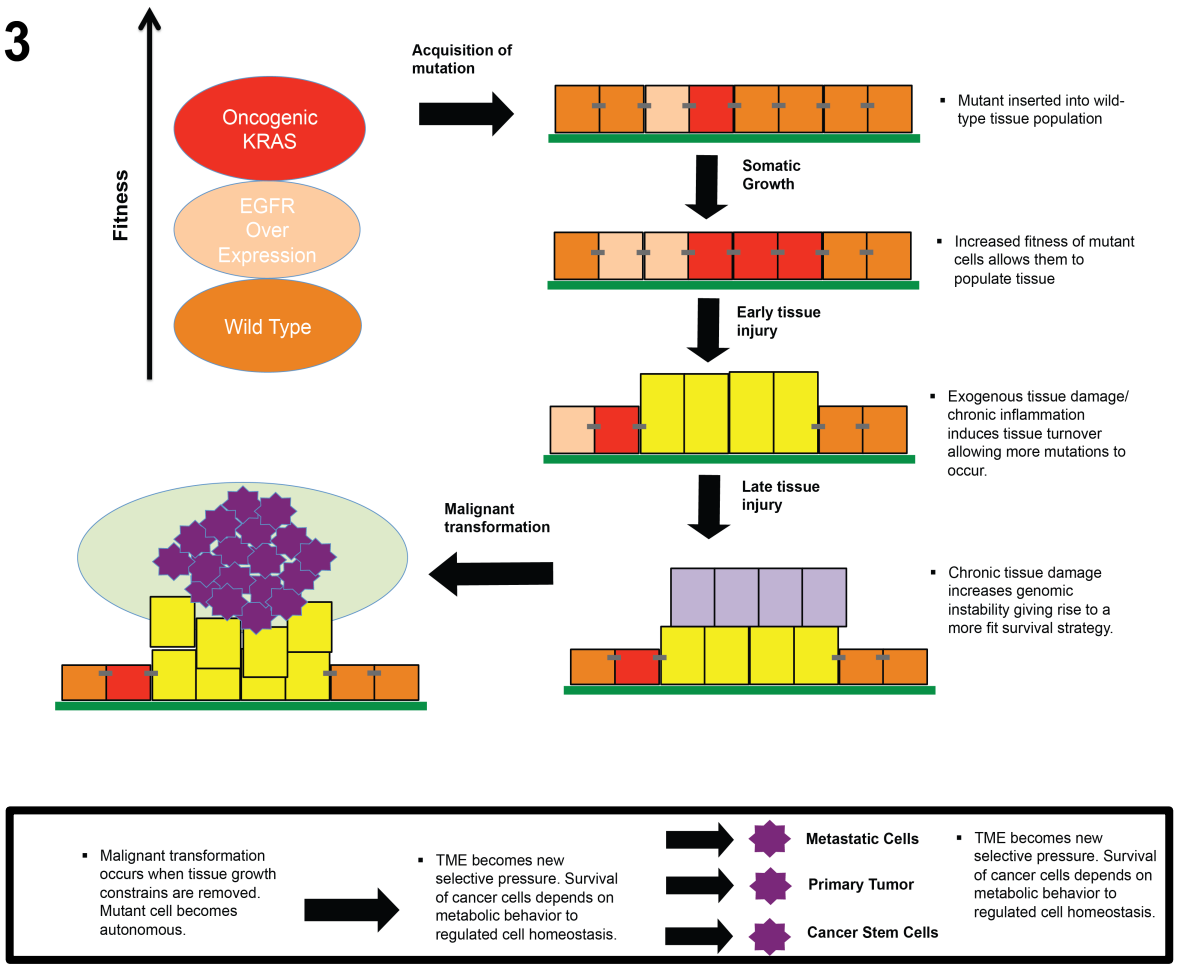

Figure 3. Schematic representation of somatic evolution of cancer. Cells with mutations in PDAC have more fitness than wild-type cells. If these mutations are gained, the more fit mutant population will populate the tissue and may gain more mutations through injury or chronic inflammation. Malignant transformation is the result of cells budding off from the tissue basal membrane into the ductal lumen and modification of TME, which is the new selective force in carcinogenesis. The bordered text indicates the subsequent tumorigenic steps of PDAC cells.

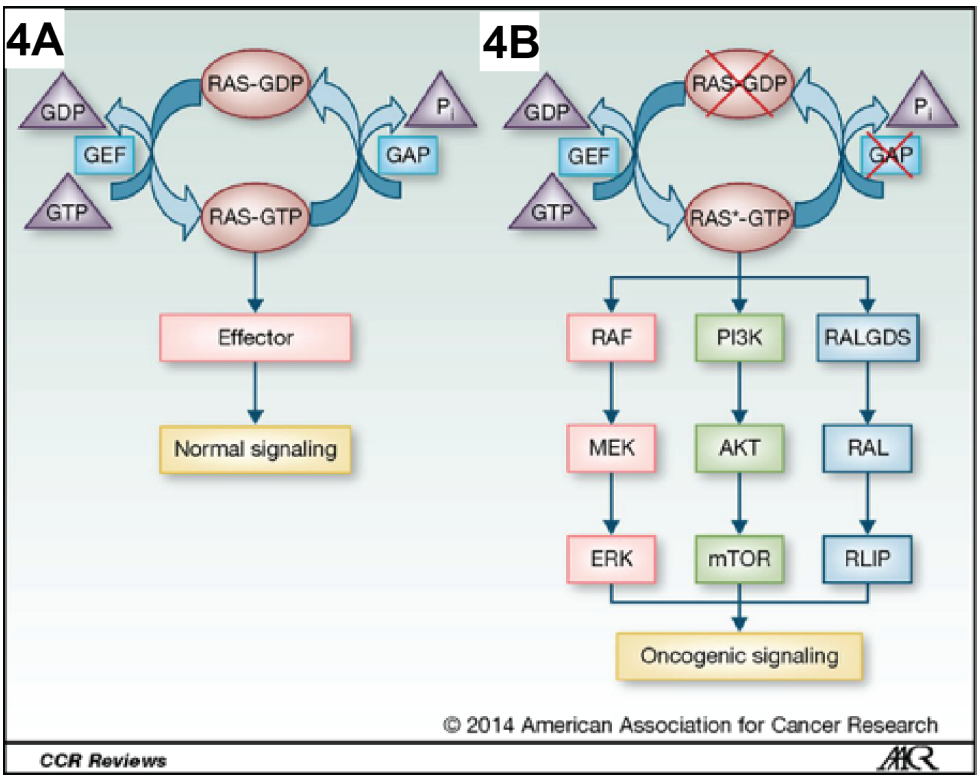

Figure 4. Oncogenic KRAS and its downstream targets. Figure 4A. Wild-type KRAS is regulated by GEF and GAP, which regulate the KRAS GTP-bound form. Figure 4B. Oncogenic KRAS induces oncogenic signaling through ERK and AKT signaling cascades. Adapted from Vasan et al. [137]

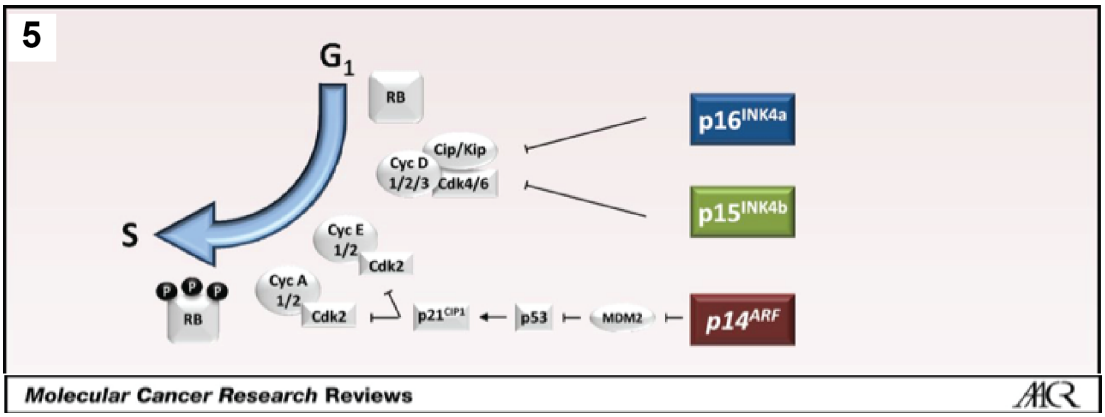

Figure 5. Function of *CDKN2A* gene products in cell cycle regulation. $p16^{INK4A}$ functions in the RB tumor suppressor pathway through inhibition of CDK4/6 activity. $p14^{ARF}$ inhibits E3 ubiquitilin ligase MDM2 thereby preventing degradation of p53. The p53 pathway and RB pathway are integral in blocking inappropriate cellular proliferation. Figure adapted from LaPak & Burd [138]

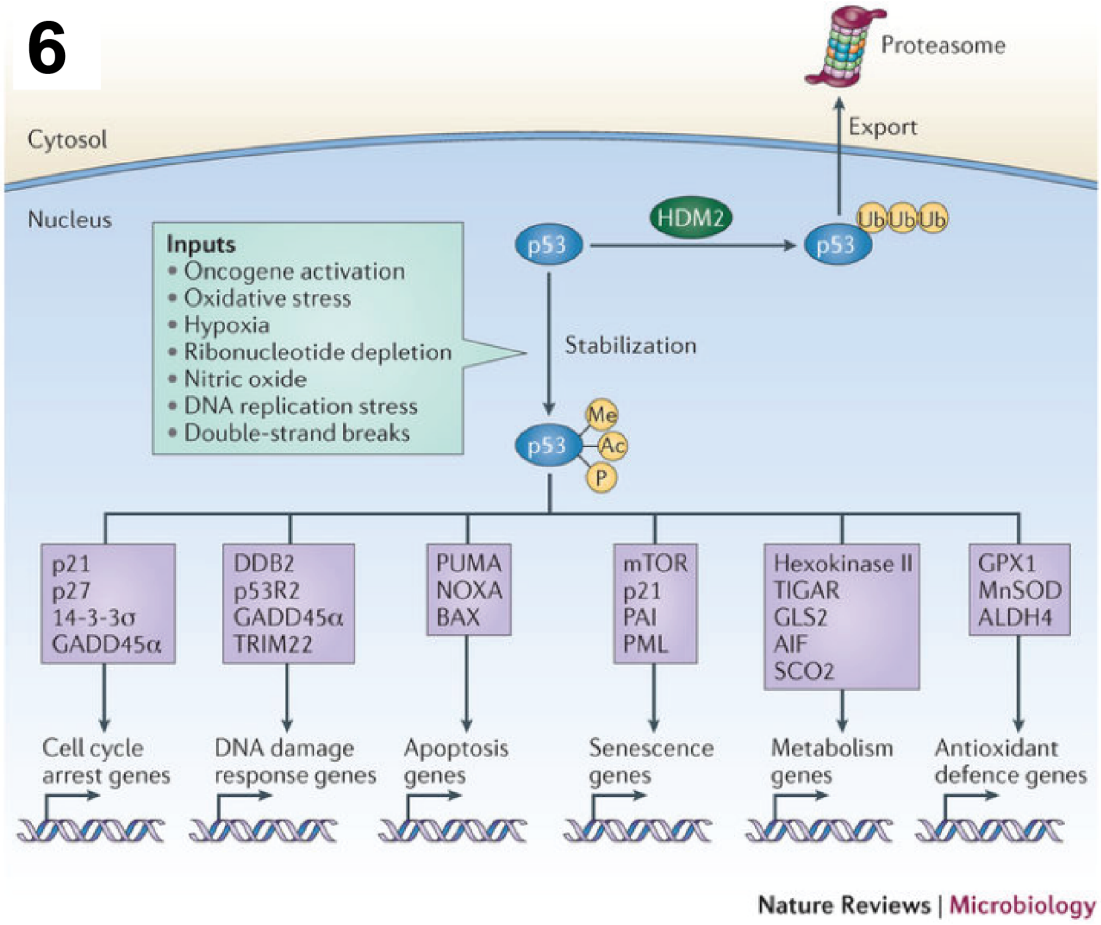

Figure 6. p53 the guardian of the genome. The tumour suppressor p53 plays a critical role in maintaining genomic stability, apoptosis mechanisms, metabolism, and antioxidant defense. p53 is polyubiquitylated by the E3 ubiquitin ligase, MDM2, leading to its proteasomal degradation. However, in response to stimuli such as oxidative stress, hypoxia, oncogene activation and DNA damage, p53 becomes post-translationally modified and stabilized to activate multiple pathways in response to cellular stress. Depending on the severity of DNA damage, p53 can induce cell cycle arrest, senescence, or apoptosis. p53 affects several metabolic pathways, including glycolysis, the pentose phosphate pathway and oxidative phosphorylation. Furthermore, p53 up-regulates antioxidant defense genes encoding reactive oxygen species (ROS)-removing enzymes that are important for cellular and genetic stability and thus contribute to the anti-tumour function of p53. Figure adapted from Siegl & Rudel [139]

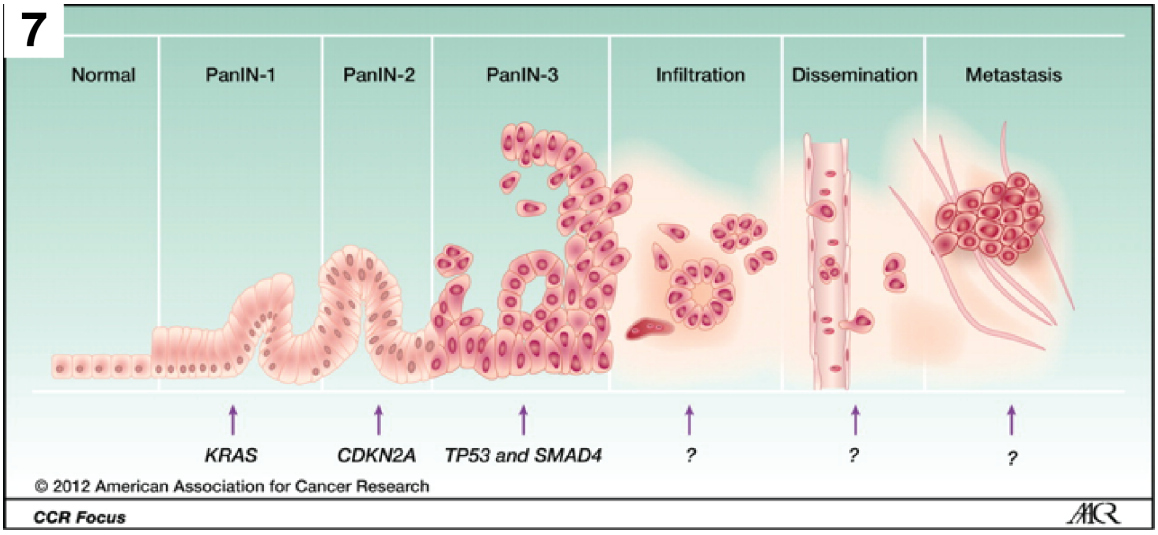

Figure 7. PDAC progression. PDAC evolves from pre-malignant well-characterized histologically evaluated pancreatic lesions, pancreatic intraepithelial neoplasms or PanINs. These pancreatic lesions are characterized by increased tissue disorganization and gaining of malignant mutations. The genetic signature of PDAC includes *KRAS* (PanIN-I) , INK4A (PanIN-II), *TP53* (PanIN-III), and *SMAD4* (PanIN-III). Figure adapted from Iacobuzio-Donahue et al. [140]

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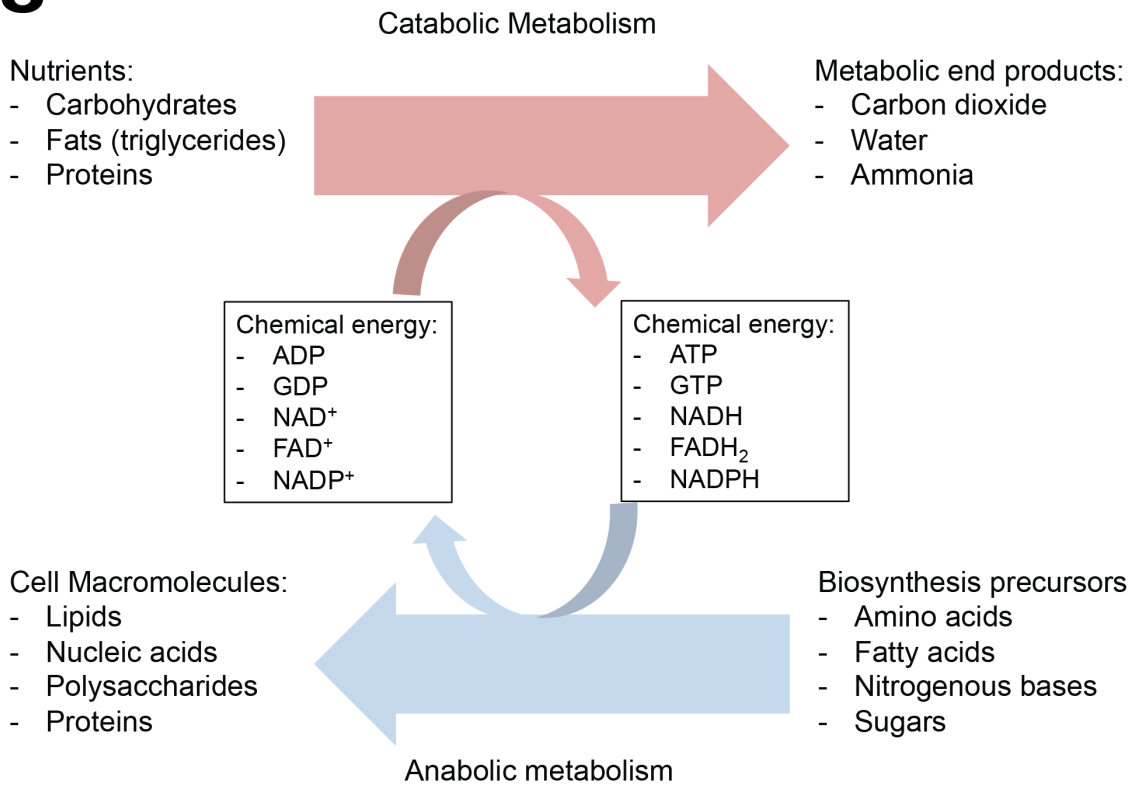

Figure 8. Summary of mammalian cell metabolism. In catabolic metabolism nutrients are broken down to produce energy yielding metabolic by products. In anabolic reactions chemical energy generated through catabolism is used to generate building blocks of macromolecules through biosynthetic biochemical reactions.

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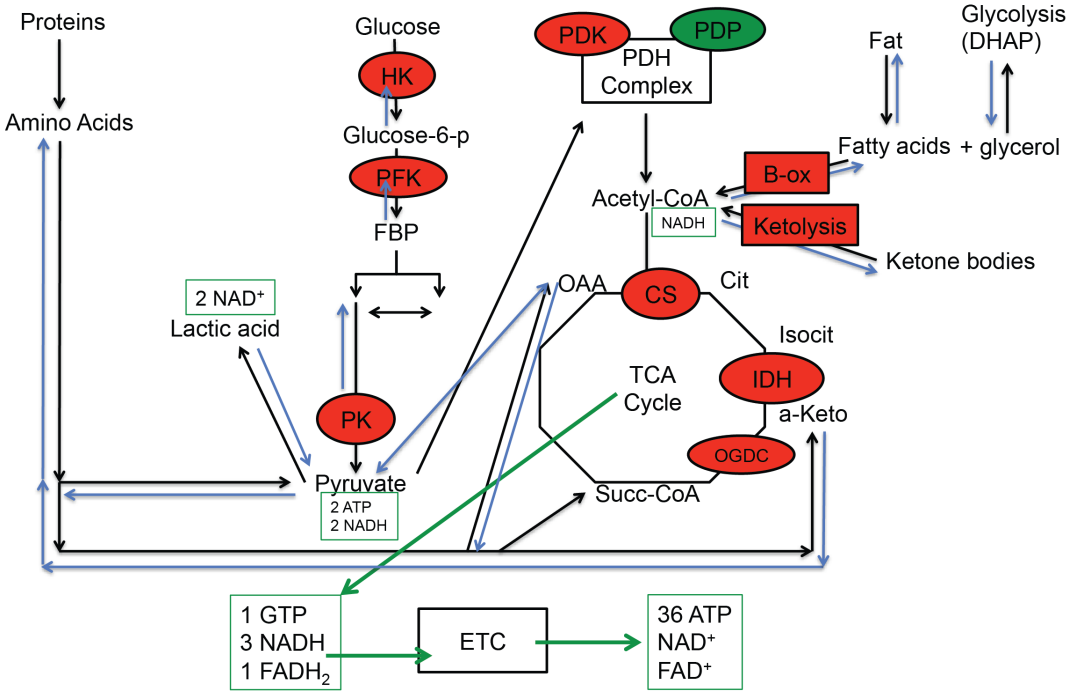

Figure 9. Canonical catabolic pathways. Glucose, amino acids, and fatty acids are the main metabolic substrates for catabolic reactions. Amino acids can be metabolized into pyruvate and/or metabolic intermediates of the TCA cycle. Lipids can enter the TCA cycle through B-oxidation, a series of reactions where acetyl-coA is released from fatty acids. Similarly glycerol can be metabolized to enter to enter glycolysis metabolism as glyceraldehyde-3-phosphate. In this figure, the red circles indicate enzymes that are regulated; 4-sided square boxes indicate complexes that are regulated. In PDH complex the enzyme inhibiting is highlighted in red and the enzyme that promotes it is highlighted in green. Blue arrow represents the reaction in the opposite direction. Green boxes and green arrows represent the chemical energy generated at the end of each pathway.

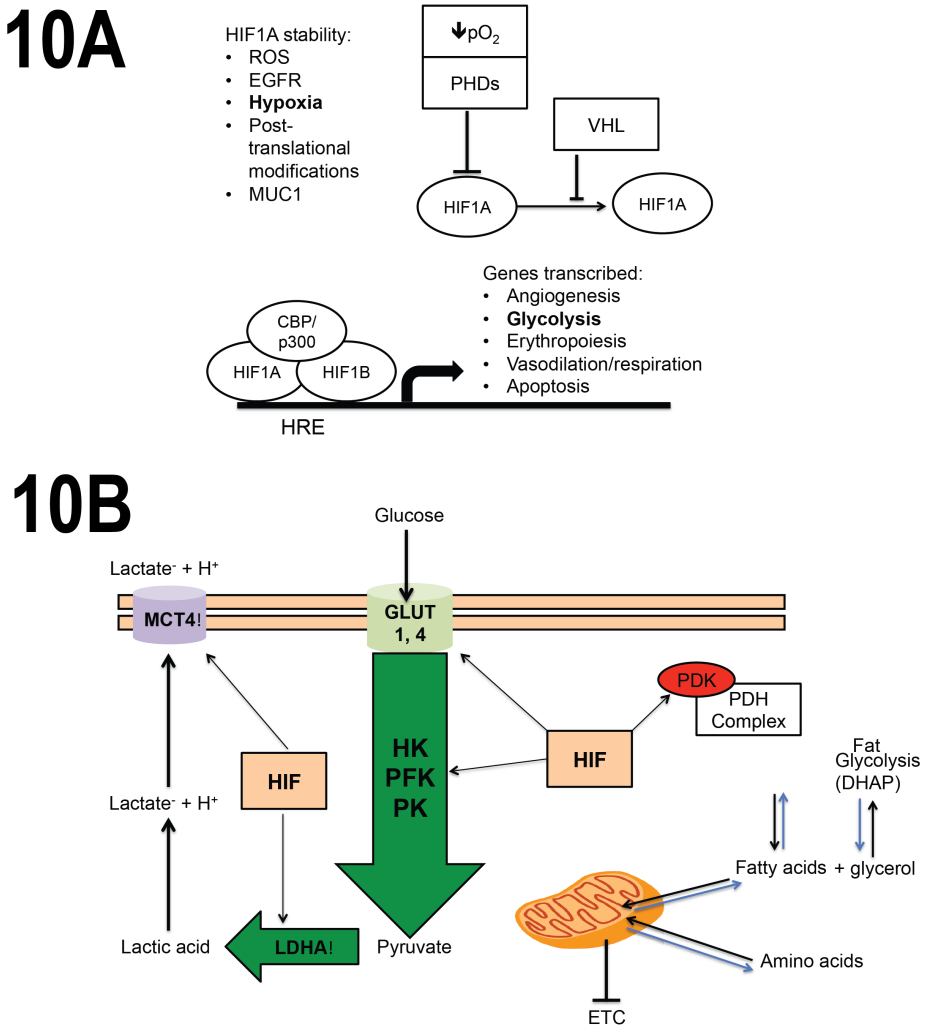

Figure 10. Hypoxia inducible response leads to metabolic reprogramming. Figure 10A. Stress factors regulate HIF1α stability through posttranslational modification enabling stability of HIF1α allowing formation of the HIF complex through interaction of HIF1α and HIF1β. The canonical mechanism of HIF1α stability is hypoxia (in bold font). Decreased oxygen levels inhibit hydroxylation of HIF1α by propyl hyrdoxylases (PHDs), which prevents its recognition by E3 ligase von Hippel-Lindau (VHL), and subsequent proteasome degradation. When stable, HIF complex binds HRE sequences to induced expression of target genes. Figure 10B. HIF target genes include glycolysis rate limiting enzymes, glucose transporters, as well as, lactic acid fermentation enzyme LDHA and MCT4 for rerelease of excess lactate. HIF also increases expression of PDK which henbits PDH complex though phosphorylation thereby uncoupling glycolysis from oxidative metabolism.

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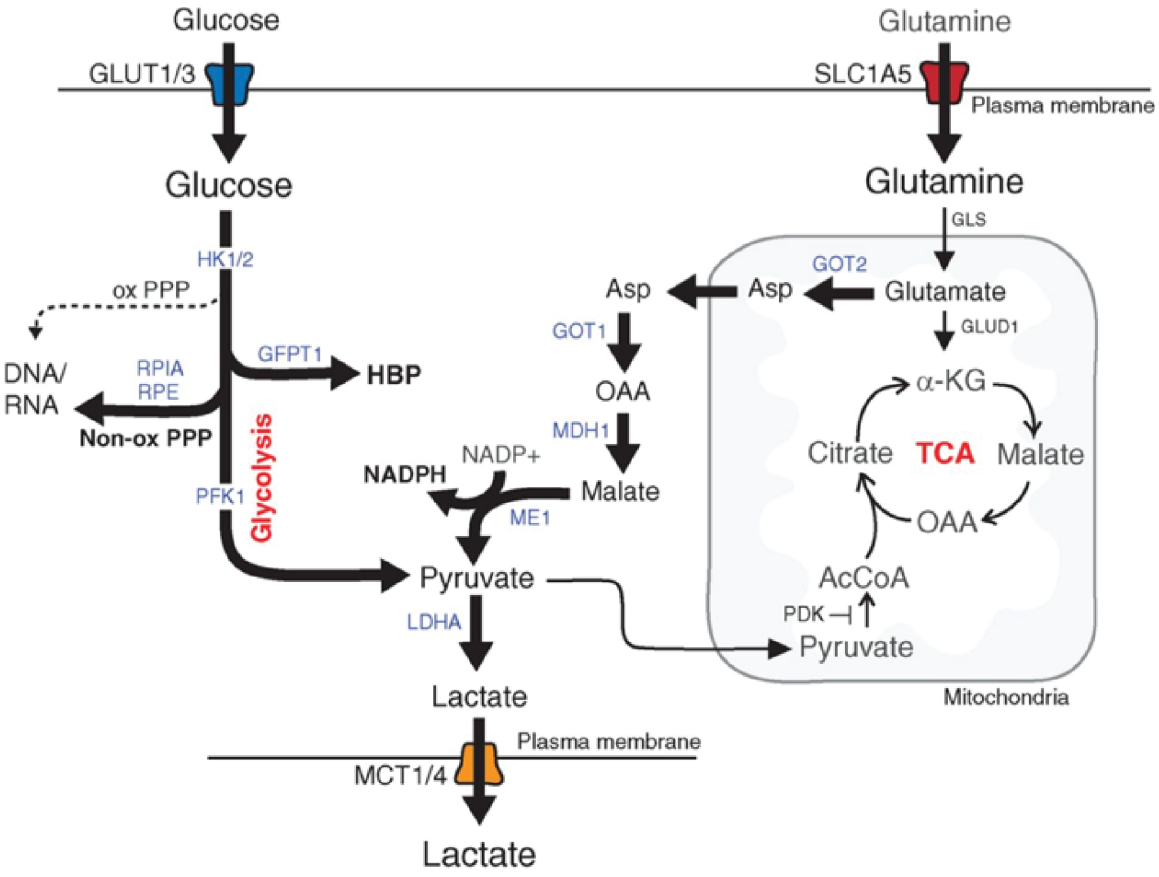

Figure 11. Oncogenic is a master regulator of PDAC metabolism. KRAS diverts glycolysis intermediates of metabolism into the HBP by inducing overexpression of GFPT1. Similarly, KRAS induced expression of RPIA and RPE to divert glycolysis intermediates to the non-oxidative PPP. KRAS also inhibits GLUD1 and overexpresses GOT1 allowing glutamine to be metabolized through the non-canonical pathway. Figure adapted from Perera & Bardeesy [141]

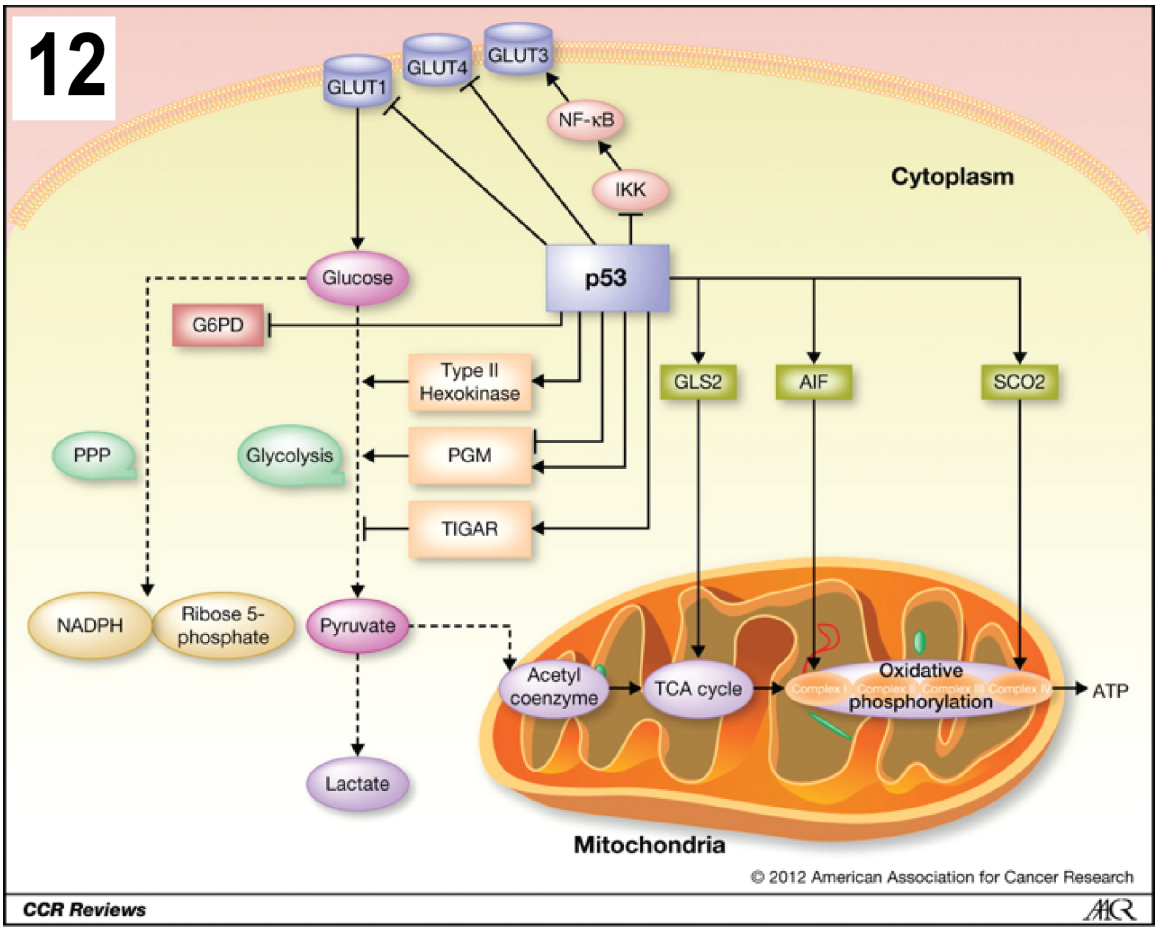

Figure 12. p53 mediated metabolic regulation. p53 regulates glucose catabolism in cancer cells through effectors such as TIGAR, PGM, GLUT1/4, IKK, HKII, SCO2, GLS2, and G6PD. These proteins work together to enhance oxidative metabolism and reduce glycolysis and PPP. Figure adapted from Shen et al. [142]

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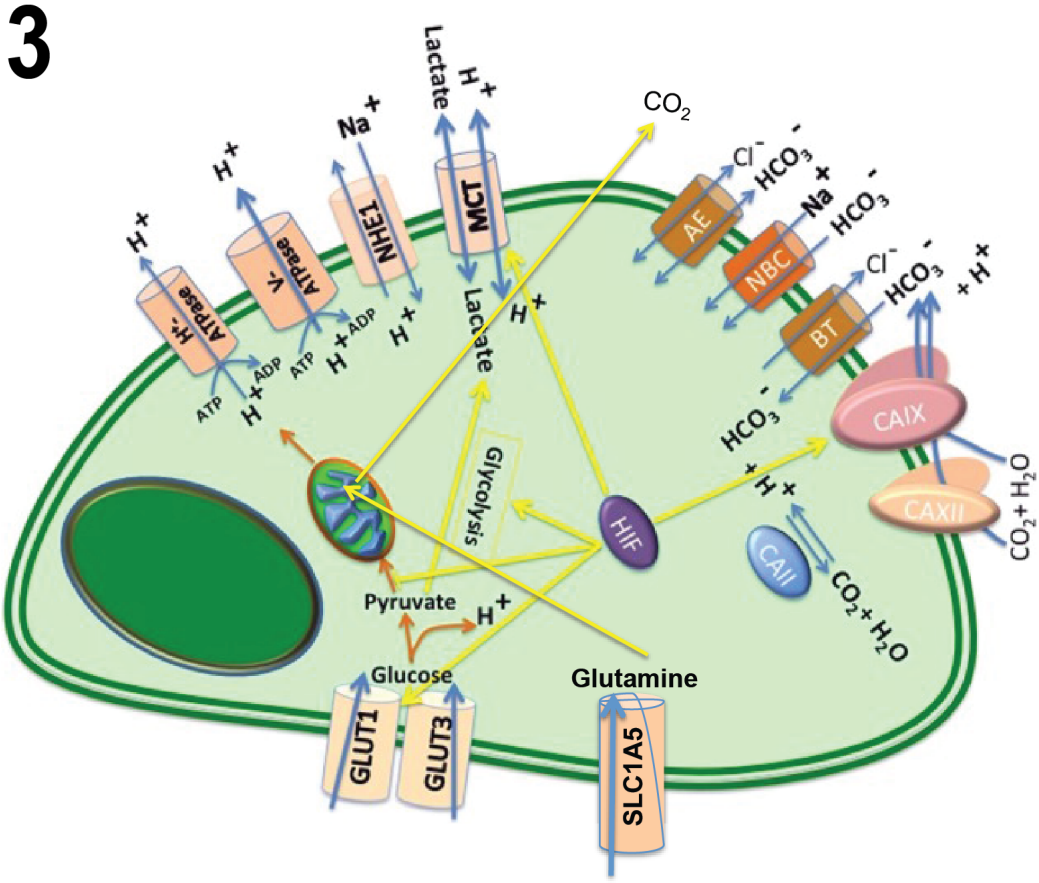


Figure 13. Major pH regulators in a cancer cell. Hypoxia and oncogenic driven Warburg effect metabolic shift to increase lactate production from glycolysis or glutamine. Similarly, reductive decarboxylation produces protons from excess CO_2 ($CO_2+H_2O \rightarrow H_2CO_3 \rightarrow H^+ + HCO_3^-$). CO_2 moves passively across the plasma membrane and is converted to carbonate by carbonic anhydrases. Protons are pumped out by MCTs, NHE, and V-ATPase; while buffer (HCO_3^-) is imported by NBCs and AEs (BT). Figure adapted from Damaghi et al. [143]

14

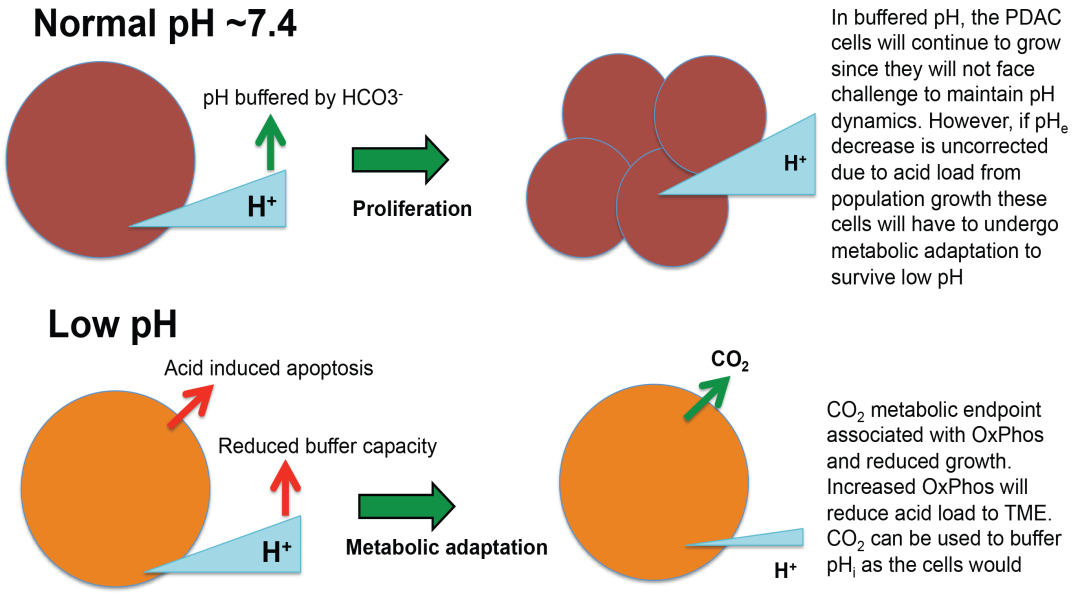


Figure 14. Hypothesis. In normal (physiological pH), PDAC cells are able to conduct Warburg effect metabolism because cellular proton load into the TME is buffered. Reduced buffering of pHe creates a significant difference between cytosolic-extracellular [H+] gradient. In this conditions release of protons from metabolic reactions of glycolysis will not be favored, as the electrochemical gradient will rather favor the inward flux of protons. Cancer cell growth is stalled at pH_i value bellow 7.2 further decrease in pH will induce apoptosis. Thus, the cells must reprogram their metabolism to diminish production of metabolic acids—lactic acid. Thus, they must switch to depend on oxidative phosphorylation to make energy for cellular homeostasis in stress conditions.

Chapter 1 Tables

Table 1 A

Disease Factor	Mutated Genes	PDAC Increased Risk
Hereditary breast and ovarian cancer syndrome	*BRCA1, BRCA2, PALB2*	2-3.5
Lynch syndrome (hereditary non-polyposis colorectal cancer)	*MLH1, MSH2, MSH6, PMS2, EPCAM*	8.6
Familial adenomatous polyposis	*APC*	4.5-6
Peutz-Jeghers syndrome	*STK11/LKB1*	132
Familial atypical multiple mole melanoma pancreatic carcinoma syndrome	*P16INK4A/CDKN2A*	47
Hereditary pancreatitis	*PRSS1, SPINK1*	69
Cystic fibrosis	*CFTR*	3.5
Ataxia-telangiectasia	*ATM*	Increased

Table 1B

Risk factor	Increased PDAC risk
Current cigarette use	1.7-2.2
Current pipe or cigar use	1.5
> 3 alcoholic drinks per day	1.2-1.4
Chronic pancreatitis	13.3
BMI > 40 kg/m^2, male	1.5
BMI > 40 kg/m^2, female	2.8
Diabetes mellitus, type 1	2
Diabetes mellitus, type 2	1.8
Cholecystectomy	1.2
Gastrectomy	1.5
Helicobacter pylori infection	1.4

Table 1. The risk of PDAC is elevated by intrinsic factors such as familial diseases associated with loss of tumor suppressor genes and the risk of PDAC is also elevated by extrinsic factors such as alcoholism and obesity. Table 1A. Intrinsic factors associated with PDAC. Table 1B. Extrinsic factors associated with PDAC risk. [144]

Table 2

Name	Abbreviation	Description
Carbonic Anhydrase II	CAII	Carbonic anhydrases catalyze the conversion of carbon dioxide, the final product of oxidative phosphorylation, and water to produce carbonic acid which deprotonates in pH_i to generate bicarbonate and protons. CAIX and CAXII are transmembrane CAs that has been identified to play roles in tumor progression and metastasis. CAIX is a target of HIF1α its expression is up regulated in hypoxic regions of tumors. Intracellular CAs such as CAII will dehydrate metabolically produced bicarbonate into aqueous CO_2 in a reaction consuming a proton thereby promoting passive transport of CO_2 [143, 145].
Carbonic Anhydrase IX	CAIX	
Carbonic Anhydrase XII	CAXII	
Anion exchanger $Cl^-/HCO3^-$	AE, SLC4 family	These transporters facilitate the movement of HCO^-_3 ions across plasma membranes to either acidify or alkalinize the pH_i [143, 146].
Sodium bicarbonate exchanger Na+/HCO3-	NBC family	
Anion transporters	SLC26 family	
Aquaporins	AQP	
Sodium hydrogen exchanger Na+/H+	NHE1	NHE1 is the most common isoform of the Na^+/H^+ exchanger. NHE1 is the most active transporter in pH_i homeostasis. It uses the sodium electrochemical gradient to extrude H^+ when the cytosolic pH becomes too acidic. NHE1 functions as a pHi sensor during intense metabolic activity. NHE1 is known to be activated by EGF signaling as well [143, 147].
Vacuolar ATPase	V-ATPase	V-ATPases pump protons out of the cytoplasm and into intracellular vesicles such as lysosomes using free energy from ATP hydrolysis [148].
Monocarboxylate Transporter 1	MCT1	MCTs transport mono-carboxylic acids such as lactate, pyruvate, and ketone bodies into and out of cells across plasma and mitochondrial membranes. Only 4 isoforms, MCT1–MCT4, have been functionally characterized as proton-linked monocarboxylate transporters. MCT1 and MCT4 are commonly overexpressed in tumors as they are essential in regulating the cytosolic efflux of lactate and protons produced through HIF or Warburg effect up-regulated glycolysis. Therefore, their expression is critical to maintain pH_i homeostasis at the expense of contributing to extracellular acidosis [145, 149].
Monocarboxylate Transporter 4	MCT4	

Table 2. Membrane transporters regulating pH_i.

Chapter 2

Results:

GOT1-Mediated Anaplerotic Glutamine Metabolism Regulates Chronic Low pH Stress in PDAC Cells

Introduction

Metabolic alterations represent an important hallmark of cancer cells [150]. Metabolic reprogramming allows cancer cells to sustain uncontrolled proliferation by rapid generation of ATP, biosynthesis of macromolecules, and maintenance of redox status [151]. Cancer cells can also reprogram the major metabolic pathways (carbohydrates, proteins, lipids, and nucleic acids) in order to meet these basic demands for uncontrolled proliferation [56, 152]. The characteristic metabolic phenotype seen in cancer cells is the Warburg effect, which operates by enhancing glucose uptake and flux into glycolysis, while simultaneously diminishing the glucose carbon flux that enters the TCA cycle in the mitochondria, even in the presence of oxygen [36, 153]. Although ATP generation through substrate level phosphorylation is very rapid, this mechanism is far less efficient than oxidative phosphorylation in generating energy from glucose. Thus, the metabolic phenotype observed in the Warburg effect demands very high glucose uptake to meet the energetic, biosynthetic, and redox needs of cancer cells. For this reasons the increased glucose uptake of cancer cells is useful for diagnosing cancer using radiolabeled glucose analog ^{18}F-fluorodeoxyglucose and positron emission tomography (FDG-PET) to image and evaluate tumor progression without the need of a biopsy [154, 155].

Because of the enhanced metabolic rate of rapidly proliferating tumor cells, the glucose that is metabolized through substrate level phosphorylation produces lactic acid as the end product. Lactic acid is a weak acid with pK_a with approximately ~pH 4, and thus, it quickly dissociates loses a hydrogen ion [156]. Lactate is transported outside of the cell by monocarboxylate symporters along with protons resulting in decreased pH in the extracellular milieu [157, 158]. Intracellular hydrogen ions can also be removed by

sodium hydrogen exchangers that import sodium ions and extrude hydrogen ions, thereby acidifying the extracellular environment [159, 160]. Similarly, vacuolar ATPases extrude hydrogen ions against their concentration gradient to the extracellular space, and hence, lower the extracellular pH [161]. *In vitro* studies have shown that rapidly growing cells, which exhibit the Warburg effect, increase the expression of these cell surface proteins to maintain an alkaline intracellular pH environment [57, 162]. Indeed, increased intracellular pH is an established permissive signal for cellular proliferation promoting survival by limiting apoptosis, a process that is associated with intracellular acidification [163, 164]. The role of low extracellular pH in carcinogenesis is thus paradoxical: on one hand alkaline intracellular pH promotes proliferation and survival, while at the same time, extracellular pH promotes invasion and metastasis at the cost of inducing stress, senescence, and apoptosis [159, 165, 166].

In addition to glucose, glutamine metabolism is also essential for the proliferation of cancer cells. Recent studies have demonstrated that glutamate derived from glutamine is utilized by highly proliferative cells to generate non-essential amino acids (NEAAs) through the glutamic-oxaloacetic transaminase enzymes (GOT1 and GOT2), while quiescent cells metabolize glutamate through GLUD1 (glutamate dehydrogenase 1) and subsequent decarboxylation reactions in the TCA cycle [167, 168]. Thus, glutamine can be metabolized through both anabolic (anaplerotic) and catabolic pathways.

Several oncogenes are implicated in reprogramming tumor cell metabolism. One such gene is *KRAS,* which upon accumulating activating mutations serves as a key signature oncogene that serves a prominent role in malignant transformation and tumor progression in PDAC [5, 169]. PDAC cells with oncogenic *KRAS* have reprogrammed glucose and glutamine metabolism to serve anabolic processes [96, 108]. Canonical

glutamine metabolism occurs through glutamate synthase (GLS)-mediated conversion of cytoplasmic glutamine into glutamate. Glutamate is then metabolized in the mitochondria through GLUD1 into alpha-ketoglutarate that enters the TCA cycle [170]. The non-canonical pathway metabolizes glutamate to aspartate and alpha-ketoglutarate through GOT2; aspartate is subsequently metabolized to oxaloacetate by GOT1 in the cytosolic compartment. Aspartate is metabolized by malate dehydrogenase (*MDH*) to malate, which is then metabolized by malic enzyme (*ME*) to produce pyruvate. These anaplerotic reactions increase the NADPH/NADP ratio thereby maintaining ROS balance. PDAC cells are dependent on these reactions for maintenance of intracellular ROS levels as it is evidenced by the decrease in cell survival upon knockdown of enzymes in the pathway.

Due to metabolic reprogramming by oncogenic KRAS present in 90% of PDAC cases, extracellular acidification is highly abundant. While the regulation of pH in cancer cells has been studied thoroughly, the metabolic adaptations to chronic low ph induced stress are not well defined. Therefore, in the current study, we investigated the metabolic basis of adaptation to chronic low pH stress in PDAC cells, which exhibit high glycolytic capacity, by subjecting them to chronic low ph. We utilized PDAC cells with oncogenic *KRAS* to identify the metabolomic alterations in PDAC cells under chronic low ph and identify vulnerabilities for therapy. Here, we report a pronounced increase in non-canonical anaplerotic glutamine metabolism, which serves the bioenergetic needs and maintains ROS balance in cells undergoing acidosis stress.

Materials & Methods

Cell lines:

Cell culture of PDAC cell lines S2-013 and Capan-1 have been described previously [93, 171]. The S2-013 and capan-1 cell lines were obtained from a liver metastases of PDAC patients. In immunodeficient nude mice orthotopic implantation, Capan-1 and S2-013 forms a tumor that produces mucin and is morphologically and biochemically similar to the PDAC tumor of origin. Capan-1 and S2-013 cell lines both bear activating *KRAS* mutation, mutant *TP53* affecting p53 DNA binding, and homozygous deletion of *CDKN2A*/p16. While, *SMAD4* has no alterations and Capan-1 have shallow deletions, cosmic database. Cells were validated by STR profiling.

Control pH culture:

Cell lines were cultured in high glucose high glutamine Dulbecco's Modified Eagle Medium (DMEM) (Hyclone) containing 4.5g/L glucose (Sigma-Aldrich) and 0.584g/L glutamine (Sigma-Aldrich). Additionally, 1mM sodium pyruvate and 5% FBS were supplemented to complete the media. To set control pH 3.7g/L $NaHCO_3$ were used, this concentration sets pH of media approximately to ~7.4 at 37 degrees Celsius, and phenol red in DMEM indicates pH changes. The media was replaced every 24hrs to maintain pH from metabolic acids produced by cell growth.

Low pH cell culture:

Cell lines were cultured in high glucose high glutamine Dulbecco's Modified Eagle Medium (DMEM) (Hyclone) containing 4.5g/L glucose (Sigma-Aldrich) and 0.584g/L glutamine (Sigma-Aldrich). Additionally, 1mM sodium pyruvate and 5% FBS were supplemented to complete the media. To set low pH conditions 1g/L $NaHCO_3$ were used, this concentration sets pH of media approximately to ~6.9-7 at 37 degrees Celsius, and phenol red in DMEM indicates pH changes. Low pH media with values 6.7 and 6.4 was prepared using 0.5 and 0.1 g/L $NaHCO_3$ respectively. The media was

replaced every 24hrs to maintain pH from metabolic acids produced by cell growth. Lactic acid was used to decrease the pH of media, but the pH was very inconsistent being very acidic without sodium bicarbonate and titration with bicarbonate or hydroxide equivalents. The organic zwitterionic organic chemical buffer HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was used as well, but it required addition of HCl equivalents to lower the pH to 7.0 and it was not maintained in the incubator. To establish chronic low pH exposure, cells were cultured in pH 6.9~7.0 continuously for 14 days using bicarbonate as the buffer of choice. The media was changed every 24 hours during this period.

pH maintenance during in vitro incubation:

The pH value of the media is critical for growth of cells in culture and should be buffered to pH 7.4 in order to replicate physiological conditions. Thus the conditions in the incubator are 20% oxygen, same as atmosphere, and 5% for CO_2 to replicate blood levels, 40mmHg. Oxygen is not a polar molecule therefore it will not dissolve in water, but CO_2 will dissolve in water forming carbonic acid. However, because the pH of 1L DMEM is 6.6 without $NaHCO_3$, carbonic acid will quickly dissociate because of its acid dissociation constant (K_a) being lower than that of the pH of the media producing bicarbonate and protons. Furthermore, Henry's law of gas solubility states that at a constant temperature of 37°C the amount of $CO_2(g)$ that dissolves in the media is directly proportional the partial pressure of $CO_2(g)$ in equilibrium with the media. Therefore, conversion of gas to aqueous CO_2 is described in the following equation, $CO_2(aq) \leftrightarrows CO_2(g)+H_2O(l)$, at constant equilibrium (K_{eq}) conversion is defined by $K_{eq}=[CO_2(g)][H_2O(l)]/[CO_2(aq)]$ and assuming that water concentration is constant we can define the equation as a dissolving constant of gas pressure, K_H, which also defines the partial pressure of the gas that is dissolved thus $K_H=K_{eq}/[H_2O]=pCO_2/[CO_2(aq)]$. In this

way the equilibrium constant can be calculated for 1M of CO_2 which is K_H=29.41 and applying this to the 5% CO_2 in the incubator which equates to 38mmHG or 0.5 ATM the amount of dissolved CO_2 gas in the incubator is $1.7x10^{-3}$M, $K_H=pCO_2/[CO2(aq)]$ then $[CO2(aq)]=pCO_2/K_H$. Therefore, $CO2_{(g)}$ gas that dissolves in the incubator will exist as bicarbonate HCO_3^-, $CO2(g)+H_2O \rightarrow H_2CO_3 \rightarrow HCO_3^- + H_3O^+$. For this reason, to replicate physiological pH of 7.4 the pH must be adjusted using buffers systems such as natural-buffer system by adding bicarbonate and/or chemical buffering using zwitterions. For this study I opted to buffer pH using the natural-buffer system (bicarbonate). The human body has a reserve of $NaHCO_3$, from kidney, and plenty of HCO_3^- circulating in the blood, which is very useful to maintain blood pH at 7.4 by removing protons using the vascular system. Protons generated from metabolic acids combine with HCO_3^- to form $H2CO_3$, because of the increased concentration of bicarbonate the reverse reaction occurs and carbonic acid is converted to CO_2 and water. Excess CO_2 in blood is picked up by red blood cells (RBC) and undergoes the following reaction $CO_2+H_2O \rightarrow H_2CO_3 \rightarrow HCO_3^-+H^+$ with the first reaction catalyzed by carbonic anhydrase (CA). This mechanism is essential for delivery of oxygen to tissues because increased protons in RBCs reduces hemoglobin binding affinity to O_2 causing its release into tissues. At the same time cells exchange HCO_3^- for Cl^-, through anion exchanger (AE), thereby increasing plasma CO_2 and maintain a high pCO_2 for O_2 delivery. In the lung, pO_2 increases and forces protons of Hb allowing O_2 to bind Hb. The accumulation of protons leads to a shift in AE and bicarbonate is imported and combines with protons to form carbonic acid that is converted to water and CO_2 by CA, $H^+ + HCO_3^- \rightarrow H_2CO_3 \rightarrow CO_2+H_2O$. The human body is an open system and CO_2 is removed as gas from lung alveoli. However, cells in an incubator are not an open system and media must be changed to maintain constant pH since buffers can only contain excess accumulation of protons not remove them. The pH of control media was set to ~7.4

using 3.7g/L of $NaHCO_3$, which dissolves in the media to form sodium ions and bicarbonate ($NaHCO_3 \rightarrow Na^+ + HCO_3^-$). The pK_a of carbonic acid is 6.4 and carbonate is 10.2 indicating that the isoelectric point is 8.3, $pI=1/2(pK_a1+pK_a2)$. Therefore, at pH 7.4 the solution contains a higher amount of bicarbonate than at pH 7.0, therefore the higher concentration of $NaHCO_3$ has more base equivalents to counter metabolic acids for a longer time. Reference: [172].

GOT1 Knockdown:

Cell transfections for producing replication-incompetent lentivirus were performed by utilizing Turbofect followed the manufacturer's protocol [93, 173]. Stable short hairpin RNA (shRNA) constructs were obtained from Sigma-Aldrich: shGOT1 (34784; CCGGGCGTTGGTACAATGGAACAAACTCGAGTTTGTTCCATTGTACCAACGCTTTTTG) and shGOT1 (34785; CCGGGCTAATGACAATAGCCTAAATCTCGAGATTTAGGCTATTGTCATTAGCTTTTTG). Cells were transfected in control pH culture conditions and after puromycin selection and knockdown validation clones were plated in low pH for 14 days to establish chronic low pH expossure. Cells were validated by STR profiling.

Metabolomics:

Polar metabolite isolation was performed as described previously [174]. In short, 0.75×10^7 cells were cultured for 24h in normal DMEM. Cells were then washed with PBS and culture medium was exchanged with fresh medium 2 hours before metabolite extraction, the pH of the media was maintained. Cells were frozen in dry ice and polar metabolites were then extracted with 80% methanol by plate scraping. Metabolite extracts were subjected to LC-MS/MS analysis using multiple reaction monitoring methods described previously [175]. Data acquisition was carried out utilizing Analyst™1.6 software (AB SCIEX) and peaks were integrated with Multiquant™ (AB

SCIEX). Peak areas were normalized to the respective protein concentrations in both culture conditions. Extraction and analysis of polar metabolites was performed three times after cells had been grown in low pH for 14-days.

Reactive oxygen species assay:

Reactive oxygen species levels were determined by using oxidation-sensitive fluorescent dye 2',7'–dichlorofluorescin diacetate (DCFDA). Control and low pH cells were seeded at 3.0×10^4 cells per well in a clear bottom black 96-well plate. After the cells adhered, the media was replaced with fresh DMEM containing 10 μM DCFDA, with or without respective treatments. H_2O_2 was used as a positive control and N-acetyl cysteine (NAC) was used as a negative control. Control and treated cells were incubated at 37°C for 30 mins. The cells were washed with PBS and 100μL of PBS was added to the wells for measuring the emission of DCFDA using Biotek Cytation3 plate reader. DCFDA was measured using an excitation of 495nm and an emission of 529nm. These experiments were repeated two times with similar results. The same experiment was repated using ROS-insensitive dye 5(6)-Carboxy-2',7'-dichlorofluorescin diacetate (CDCFDA) as a negative control and H_2O_2 as a positive control.

Growth Kinetics:

To determine growth kinetics, 2.5×10^5 cells were seeded in 12-well tissue culture plates in control and low pH media. These cells were trypsinzied at 24, 48, 72, and 96 hours after cells were attached to the plate. If cells received treatment, this was re-applied every 24 hours. Once collected, cells were stained with trypan blue (Gibco) and counted using BioRad TC20 automated cell counter.

Colony formation assay:

Cells were seeded in 6-well tissue culture plates at 200 cells per well or in 12-well plates at 50 or 100 cells per plate. Cells were allowed to attach to the plate for 48 hours and then media replaced with fresh media every 72 hours. Colonies were allowed to form for 14 days. Colonies formed at the end of experiments were washed, fixed in methanol, and stained with 0.4% crystal violet in 25% methanol. Colonies containing >50 cells for each well were counted. This experiment was repeated with 100 and 200 cell numbers showing similar results.

Cell cycle analysis:

Cell were seeded at 20% confluence in 60mm tissue culture plates and allowed to adhere to the plate overnight. Cell growth was synchronized using the double thymidine block previously described [176]. After synchronization, cells were cultured in fresh media for approximately 20 hours before collection for cell cycle analysis. Cells were fixed with cold ethanol and stained with Telford reagent (16.81mg EDTA, 13.4 mg of RNAse A, 25mg of propidium iodide, and 500μL of Triton X-100, dissolved in 500mL of milli-q water). These samples were submitted for analysis to the University of Nebraska Medical Center flow cytometry research facility. This experiment was repeated twice yielding similar observations.

Glucose/Glutamine Uptake:

Cells were seeded at a density of 5×10^4 cells per well in 24-well plates and allowed to adhere overnight before conducting the experiment. Cells were cultured in glucose and glutamine, and pyruvate containing DMEM and 5% fetal bovine serum. Cells were seeded in eight replicates 3 for background, 1 for counting (normalizing), and 4 for radiolabeled metabolite uptake. For the glucose uptake assay the cells were starved for 2 hours in DMEM without glucose, glutamine, pyruvate, and fetal bovine

serum. After 2 hours the background wells were supplemented with 50x the normal concentration of glucose for 20 minutes. Control and low pH cells were then incubated with tritiated [^{3}H]2-Deoxy glucose, washed with PBS, and lysed with 1% SDS. The lysates were counted for [^{3}H] by a scintillation counter. For glutamine uptake assay, cells were incubated with [^{3}H] glutamine, but background cells were not. Cells were subsequently washed with PBS, lysed with 1% SDS and lysates were counted for [^{3}H] by a scintillation counter. These experiments were repeated three times with similar results.

Lactate Release:

Control and low pH cells were seeded in 24-well plates at 5×10^4 cells. After 12h, the culture medium was replaced with fresh phenol red free DMEM set to pH 7.4 and pH 7.0. Cells were cultured for 24h and the culture supernatants were then used to determine lactate release by lactate assay kit (Eton Bioscience Inc., San Diego, CA, USA), per manufacturer protocol. This experiment was repeated twice yielding similar observations.

ATP assay:

Cells were seeded at 5×10^5 cells per well in a 12-well tissue culture plate. The cells were maintained in control and low pH DMEM. After 24 hours, cellular ATP levels were determined using an ATP assay kit (Roche, Indianapolis, IN, USA) following the manufacturer's protocol. Values were normalized to the cell counts. This experiment was repeated twice yielding similar observations.

Cytotoxic assays:

Cells were seeded at 5×10^3 in 96-well plates or at 2.5×10^5 in 12-well plates. Cells were treated for 72 hours after they attached. The MTT assay was used in 96-well plate

culture and trypan blue stain and counting were used for cells in 12-well plate culture. GraphPad Prism statistical software was used to calculate inhibitory concentration. These experiments were repeated three times with similar results.

Quantitative real-time PCR:

Cells were cultured in 60mm dishes for RNA extraction using Trizol (Life Technologies) and cDNA was prepared using Thermo Scientific Verso cDNA Kit following manufacturer's protocol. RT-qPCR was performed using Roche FastStart Universal SYBR Green Master Mix (Roche) following the manufacturer's protocol. These experiments were repeated three times with similar results.

Growth Kinetics:

To determine growth kinetics, 2.5×10^5 cells were seeded in 12-well tissue culture plates in control and low pH media. These cells were trypsinized at 24, 48, 72, and 96 hours after the cells attached to the plate. If cells received treatment, this was re-applied every 24 hours. Once collected, cells were stained with trypan blue (Gibco) and counted using BioRad TC20 automated cell counter. These experiments were repeated three times with similar results.

Data analysis:

Student's t-test was used to compare data between two groups. Two-way ANOVA was used to compare data from multiple groups defined by two variables. When results are significant, post-hoc analyses with Bonferroni method for multiple comparisons were conducted. All data analyses were conducted using Graphpad Prism 5.

Results

PDAC cell growth is diminished in low pH conditions

Intracellular pH value is known to have a significant role in conveying proliferation and death signals [57]. For example, it has been observed that proliferating cells require an intracellular alkaline pH value greater than 7.2, to allow for growth-factor stimulated cells to enter the S-phase of the cell cycle at a faster rate, and proceed to the G_2 and M phases more rapidly [177, 178]. Furthermore, a higher pH is known to suppress mitotic arrest due to activated DNA damage checkpoints; therefore, maintaining an alkaline intracellular pH enhances bypassing of cell cycle checkpoints allowing cells to have unrestricted proliferation [179, 180]. While intracellular acidic pH promotes pro-apoptotic *BAX* by enhancing conformational changes that facilitate mitochondrial insertion thereby increasing pore formation allowing increased permeability to the mitochondria and release of pro-apoptotic proteins such as cytochrome-c into the cytosolic compartment [181]. PDAC cells in culture (pH 7.4) exhibit the Warburg Effect [93]. Hence, we investigated whether chronic low ph of extracellular milieu would have an effect on cell growth. To address this question we determined the growth kinetics of PDAC cell lines (S2-013 and Capan-1) in various acidic pH values of tumors reported in the literature and identified that the pH value between 6.9-7.0 resulted in significant growth reduction when compared to the physiological pH (Fig. 1A). To determine if reduced growth in low pH is due to reduced clonogenicity we conducted colony formation assays and identified that cells in low pH culture have reduced clonogenicity compared to the cells that are cultured in control pH (Fig. 1B). Subsequently, we synchronized growth of cells cultured in control and low pH conditions with a double thymidine block and released them for 20 hours before collecting them to be fixed and stained with propidium iodide, to determine

DNA content using flow cytometry. We identified that cells in the G_1/G_0 phases are significantly increased in low pH culture; we further observed decreased percentage of cells in the S and M phases (Fig. 1C). This data indicates cell cycle arrest in the G_1/S transition in PDAC cells in the low pH environment that results in reduced rate in cell cycle progression and growth.

Reduced glucose uptake and metabolism in low pH conditions

Rapid growth and progression through the cell cycle is associated with up-regulation of glycolysis. We thus performed [^{3}H] glucose uptake assays and determined that cells in low pH culture have a significant reduction in glucose uptake compared to cells in control pH (Fig. 2A). We also conducted a lactate release assay and found that in chronic low pH exposure there is a significant reduction in lactate release (Fig. 2B). To determine if the reduction in glucose uptake and lactate release was due to a decrease in glycolysis, we isolated polar metabolites from cells in both control and low pH culture, and conducted liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS)-based metabolomics analyses. Our LCMS analysis demonstrated that glycolysis metabolites are significantly reduced in low pH (Fig. 2C). Collectively, our data demonstrates a clear departure from the classic Warburg Effect metabolic phenotype.

Increased oxidative phosphorylation in low pH conditions

Our metabolomics data also demonstrated that cells in low pH have active mitochondrial metabolism (Fig. 3A). As a parallel nutrient source, glutamine is essential in oxidative metabolism because it can be metabolized to generate alpha-ketoglutarate and enter the TCA cycle [170]. To evaluate if alterations in glutamine metabolism complement for the reduced glucose metabolism, we next evaluated glutamine uptake. We supplemented cells with [^{3}H] glutamine and observed that cells in chronic low ph

stress have a significant increase in glutamine uptake (Fig. 3B). To determine if the increase in glutamine uptake had an effect on oxidative metabolism and ATP generation we collected cell lysates from cells cultured in low pH and control pH conditions, and observed that cells in low pH generate significantly more ATP than cells in control pH culture (Fig. 3C). Next, we treated both control and low pH cells with oligomycin, an inhibitor of complex V (ATP synthase) in the electron transport chain, which acts by blocking the channel formed by the F_0 complex of ATP synthase inhibiting H^+ movement down its concentration gradient, thereby preventing ATP synthesis [182]. Treatment of cells in control and low pH with oligomycin showed that cells in low pH are much more sensitive than cells in control pH, by several orders of magnitude (Fig. 3D). These data demonstrate that chronic low ph influences a metabolic shift that enhances glutamine metabolism over glucose metabolism resulting in a change in ATP generation from substrate level phosphorylation to oxidative phosphorylation.

Increased glutamine metabolism in low pH conditions

Metabolomic analysis identified a significant increase in the levels of metabolites involved in non-canonical glutamine metabolism in S2-013 and Capan1 cells cultured under chronic low pH (Fig. 4A). Furthermore, RT-qPCR analysis demonstrated increased mRNA levels of genes involved in non-canonical anaplerotic glutamine metabolism under chronic low ph conditions (Fig. 4B). To further validate these observations, cells in control and low pH were treated with metabolic inhibitors targeting canonical and non-canonical glutamine metabolism. Cells were treated with aminooxiacetic acid (AOA) and epigallocatechin gallate (EGCG) to inhibit the transaminase enzymes (*GOT1*, *GOT2*) and *GLUD1,* respectively. We determined that the cells in low pH were at least an order of magnitude more sensitive to AOA treatment (Fig. 4C); however, the inhibitory concentration of EGCG was only modestly different in

control and low pH (Fig. 4D). Hence, our data indicate that cells with chronic low ph develop a metabolic phenotype that is highly dependent on anaplerotic glutamine metabolism, as demonstrated by increased metabolite levels, enzyme transcription, and increased sensitivity to a transaminase inhibitor (Fig. 4E).

GOT1 counters ROS production under low pH conditions

Anaplerotic glutamine metabolism can provide the fuel for proliferation by generating ATP through the TCA cycle, generation of NADPH for redox reactions, and production of NEAAs used in protein biosynthesis [108, 168, 170]. Thus, we speculated that up-regulation of anaplerotic glutamine metabolism is required for maintenance of cellular homeostasis during stress induced by chronic low ph. Based on our metabolomics analysis, we predict that due to the low glycolytic rate observed in chronic low ph the flow of metabolites into the pentose phosphate pathway for generation of glycolysis derived NADPH is reduced. Therefore, anaplerotic glutamine metabolism is essential for ROS suppression in low pH. Furthermore, we identified increased *GOT1* levels in low pH and as previously stated *GOT1* is the first enzyme in the glutamine anaplerotic pathway and its metabolic reaction allows glutamate-derived aspartate to be metabolized and generate NADPH for ROS suppression. To determine this, we generated stable knockdowns of *GOT1* (Fig. 5A). Recent reports have shown that *GOT1* and glutamine reprogramming are increased in PDAC [183]. Of note, under control pH conditions, we observed minimal growth inhibition in *GOT1* knockdown cells, compared to control shRNA-transfected cells. However, *GOT1* knockdown cells demonstrate significant growth inhibition compared to control cells in low pH (Fig. 5B-C). The most prominent PDAC phenotype we have observed in low pH culture is the increase in oxidative metabolism, which can lead to a substantial increase in reactive oxygen species (ROS). ROS are diverse in their functions, and depending on their concentration

they may have different outcomes [184]. For instance, low concentrations of ROS may induce proliferative signaling and activation of survival pathways, but at high levels, there is ROS-induced pathology due to damages in DNA, proteins, and lipids, as a result, oxidative damage may result in growth inhibition, senescence, and cell death [185-189]. We measured ROS levels (HO, 1/2 O_2, H_2O_2) using 2,7-dichlorofluorescin diacetate (DCFDA) and found that *GOT1* knockdown cells in low pH have significantly higher levels of ROS than control cells in alkaline pH (Fig. 5D). To further validate these observations we measured intrinsic ROS levels and ROS levels induced after hydrogen peroxide supplementation using DCFDA. We utilized the ROS-insensitive dye 5(6)-Carboxy-2',7'-dichlorofluorescin diacetate (CDCFDA) as a negative control and H_2O_2 as a positive control (Fig. 5E). Of note, the inhibition of non-canonical glutamine metabolism by *GOT1* knockdown results in increased ROS levels, which are further increased in low pH culture conditions. We conclude that cells in low pH generate more ROS that are increased upon inhibition of anaplerotic glutamine metabolism due to decreased *GOT1*.

Cancer cells are known to have increased expression of enzymes that suppress high ROS levels to prevent senescence and/or apoptosis [190-192]. ROS production can be increased by oxidation of nicotinamide dinucleotide phosphate (NADPH) by NADPH oxidase enzymes (*NOX*). It can also be increased through mitochondrial electron leakage, generating increased superoxide levels [151]. Superoxide levels are reduced by superoxide dismutases (*SOD*) that can combine superoxide radicals with water to produce hydrogen peroxide (H_2O_2), which in turn is capable of initiating redox biology by oxidizing cysteine residues of proteins and initiate signaling events [193]. By LC-MS/MS-based quantification, we analyzed the redox status of the most abundant antioxidant molecules NADPH and GSH. Our analysis revealed that the NADP/NADPH ratio is very similar in both alkaline and acidic pH conditions, but the glutathione disulfide/glutathione

(GSSG/GSH) is much higher in cells cultured in low pH (Fig. 5F). These data indicate that the antioxidative capacity of GSH is lesser in low pH. In rapidly proliferating cells, NADPH is mainly produced from glycolysis in the pentose phosphate pathway, with smaller contributions from isocitrate dehydrogenase and malic enzyme [151]. Our RT-qPCR analysis of the enzymes involved in anaplerotic glutamine metabolism showed that the malic enzymes *ME1* and *ME2* have a significant increase in transcription in low pH (Fig. 4B). We also performed RT-qPCR of the enzymes involved in antioxidant metabolism, and identified a very prominent increase in the expression levels of *NOX1*, *NOX2*, and *NOX3* in *GOT1* knockdown cells cultured in low pH, in comparison to control shRNA-transfected cells (Fig. 5G-I). Of note, these transcript level differences are not compensated by an increase in *SOD1/SOD2* mRNA levels (Fig. 5J-K). Therefore, cells with *GOT1* knockdown are unable to withstand low pH microenvironment due to generation of cytotoxic ROS levels.

Oxaloacetate can rescue GOT1 knockdown cells under low pH

The reaction carried out by *GOT1* uses aspartate as a substrate and converts it into oxaloacetate (OAA) [108]. Hence, we supplemented knockdown cells in low pH with oxaloacetate to re-establish anaplerotic glutamine metabolism and allow knockdown cells to suppress ROS. As a positive control of ROS suppression, we supplemented cells with N-acetyl cysteine (NAC; a ROS quencher), and aspartate and alanine, the non-essential amino acid products of this pathway, were used as negative controls. The growth of cells in low pH was compared to the cells supplemented with 3mM of NAC, 2mM of OAA, or 0.1mM of NEAA for 96 hours. Our results indicate that both NAC and OAA enhance cell growth and colony formation in low pH under *GOT1* knockdown conditions, while supplementation with NEAA had no effect (Fig. 6A-B). To determine if OAA treatment has the same effect as NAC in decreasing ROS levels we supplemented

cells with OAA, NAC, and H_2O_2 to subsequently measure ROS levels using DCFDA. Our results showed that only the cells with *GOT1* knockdown cultured in low pH have decreased ROS levels when supplemented with 2mM of OAA (6C). Thus, our data indicates that inhibition of anaplerotic glutamine metabolism by removing *GOT1* results in increased ROS levels and the addition of OAA rescues cell growth by resuming this metabolic pathway.

Discussion

Acidification of the tumor microenvironment is a common feature of PDAC (and of most epithelial tumors). Understanding the metabolic changes that PDAC cells undergo due to acidosis stress is extremely important in designing more effective treatments. Our metabolomic analysis shows that cells in low pH depart from Warburg effect metabolism, and that they increase anaplerotic glutamine metabolism to allow cells to generate vast amounts of ATP, which in turn allows for maintaining cellular homeostasis during acidosis stress. We demonstrate for the first time that anaplerotic glutamine metabolism-mediated countering of ROS levels serves as the survival mechanism for pancreatic cancer cells under chronic low ph.

Glucose and glutamine are the primary nutrients for cancer cells; however, only glutamine can provide both carbon and nitrogen [194]. Indeed, glutamine is an important growth signal [195]. Furthermore, glutamine can be metabolized into products such as nucleic acids, glucosamine, and NEEAs [195]. Based on our experiments to determine cell growth in low pH and rescue of cell survival with oxaloacetate upon GOT1 depletion, we believe that utilization of glutamine under low pH stress is not meant to induce growth signals or biosynthesis of macromolecules instead it is used for energy

biosynthesis to maintain homeostasis through moderation of high ROS levels stressing the cells.

Recent studies have demonstrated the advantages of non-canonical glutamine metabolism over the canonical pathway. In the study conducted by Coloff et al, it was demonstrated proliferating cells could take advantage of metabolism through the transaminase enzymes, whereas, quiescent epithelial cells have decreased transaminase expression; furthermore, glutamine metabolism is diverted to *GLUD* diminishing the biosynthetic potential of glutamine metabolism [168]. Similarly, in the study by Son et al. it was demonstrated that oncogenic KRAS plays a significant role in glutamine metabolic reprogramming in PDAC through the transcriptional upregulation of GOT1 and inhibition of GLUD1 expression [108]. Furthermore, this and other studies reveal the role of non-canonical anaplerotic glutamine metabolism in the generation of NADPH and possibly ROS regulation through coupling with other redox balance pathways such as glutathione synthesis [108, 196]. Here, we have shown that PDAC cells have the potential to reprogram metabolic pathways allowing them to maintain homeostasis in acidosis stress conditions.

In our studies, the increase of ROS levels is primarily due to leakage in mitochondrial superoxides from up-regulation of oxidative metabolism during low pH stress and also from up-regulation in the transcription of NADPH oxidases. For this reason, PDAC cells develop increased ROS levels that result in reduced proliferation during low pH stress. Furthermore, we find that inhibition of anaplerotic glutamine metabolism results in an increase in ROS levels and a further reduction in proliferation (Fig 5-6). Glutamine metabolism has the capacity of generating carbon, nitrogenous sources, and NADPH for redox balance [108, 168]. Since various studies have shown that there is increased expression of the transaminase enzymes driving non-canonical

glutamine metabolism in PDAC due to oncogenic KRAS, we predict that oncogenic KRAS plays a significant role in metabolic reprograming in low pH stress. The pH of the tumor microenvironment is heterogeneous while our experiments maintained a homogeneous pH value, there may be additional differences in the metabolic phenotype that correlates with the pH value inducing cellular stress we have seen here. Our findings may have implications to future therapeutic approaches since we have discovered the metabolic pathways the extracellular pH of the tumor microenvironment can modulate in pancreatic cancer cells. Our data may provide new targets to synergize with other known therapies for pancreatic cancer and increase therapeutic effectiveness against PDAC.

Chapter 2 Figures

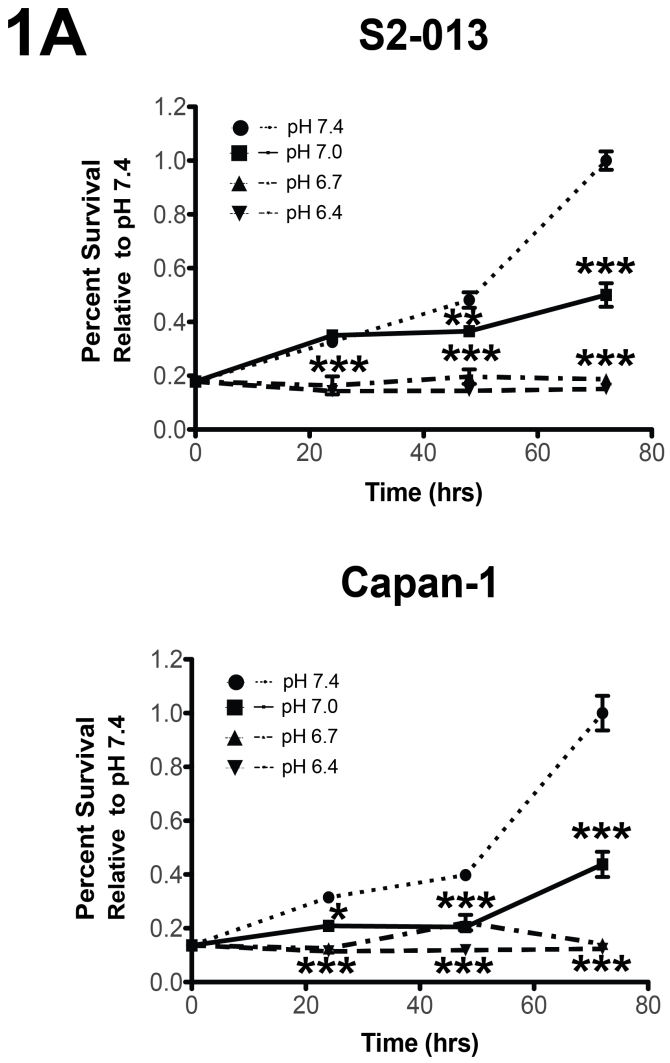


Figure 1A. PDAC cell growth is inhibited with decrease in pH of DMEM medium. (A) Survival of S2-013 and Capan-1 PDAC cells cultured under conditions of varying pH of culture media by MTT assays. These growth curves show that cells are not viable in pH values bellow 7, which was then used to establish chronic low ph. Fig. 1A values were normalized to control pH 7.4 (physiological pH value) at 72 hours of growth. Error bars represent mean ± S.E.M. from at least three different samples. Two-way ANOVA with Bonferroni post-test analysis was used for Fig. 1A to compare growth in low pH versus physiological pH.

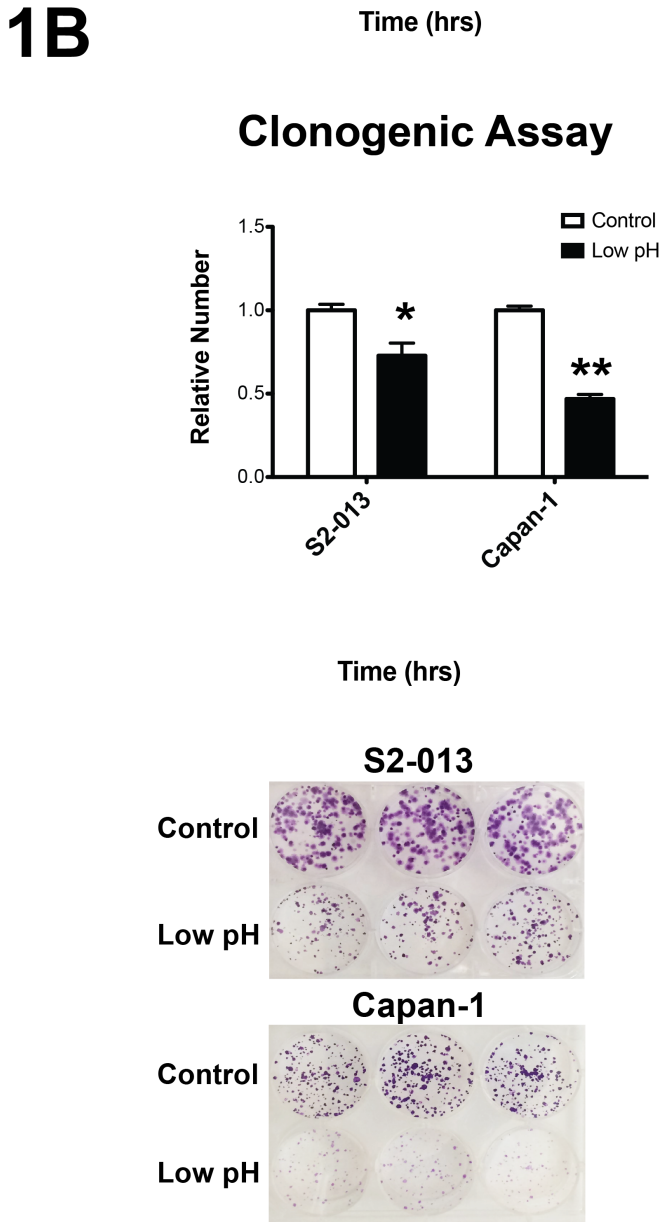


Figure 1B. PDAC cells have reduced clonogeneicity in chronic low pH. PDAC cell lines were adapted for 14 days to chronic low ph. Subsequently, these cells were sub-cultured and a fraction was diluted to seed 200 cells in 6-well plate for 14 days in control pH (7.4) and low pH (7.0). Cell numbers were normalized to that of the control pH 7.4 (physiological pH value). Error bars represent mean ± S.E.M. from at least three different samples. A two-tailed Student's t-test was used to compare low pH and control pH with p-values *p < 0.05, **p < 0.01, and ***p < 0.001.

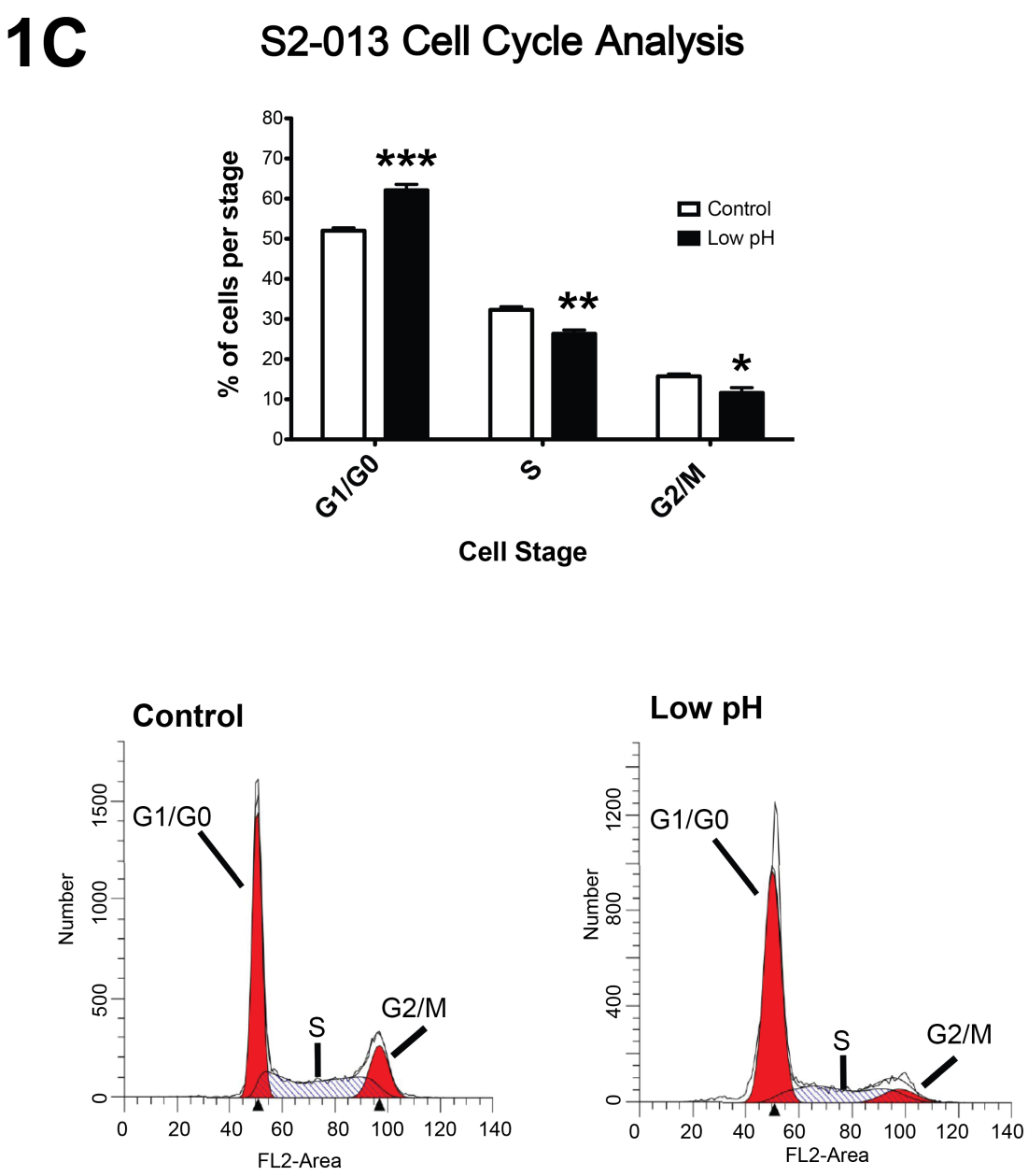


Figure 1C. Chronic low pH expossure decreases cell cycle progression of PDAC cells. Cell cycle analysis of S2-013 cells in control and low pH shows a significant increase of population in G1/G0 indicating reduced clonogeneic rate. Cell numbers were normalized to that of the control pH 7.4 (physiological pH value). Error bars represent mean ± S.E.M. from at least three different samples. A two-tailed Student's t-test was used to compare low pH and control pH with p-values *p < 0.05, **p < 0.01, and ***p < 0.001.

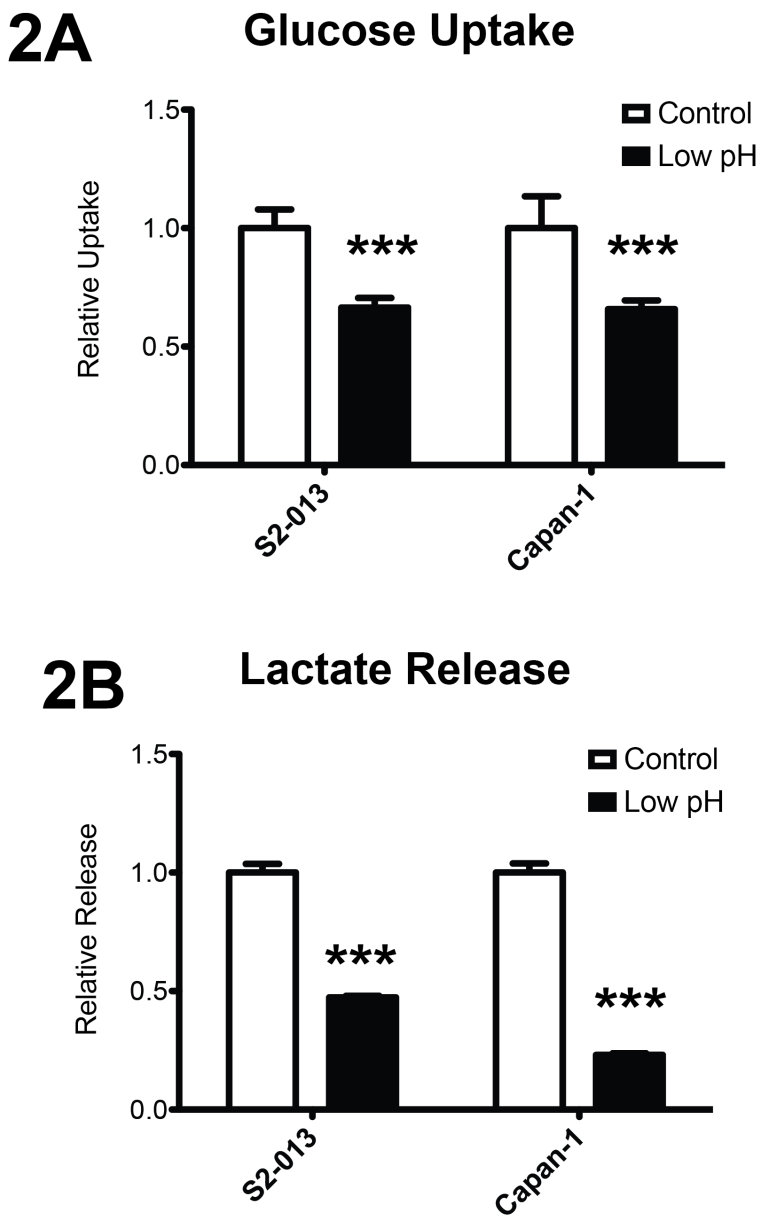


Figure 2A and 2B. PDAC cells in chronic low pH exposure shows a significant decrease in hallmarks of Warburg effect metabolism. Compared to control cells, cells cultured in chronic low pH have a significant decrease in glucose and lactate release both of which are the quintessential hallmarks of Warburg effect metabolism as shown by (A) ^{3}H-glucose uptake and (B) lactate release using colorimetric assay and LC/MS/MS analysis of extracellular metabolite extracts. Data is normalized to that of the cells at control pH (7.4). Error bars represent mean ± S.E.M. from at least three different replicates. A two-tailed Student's t-test was conducted to compare uptake/release in low pH relative to control pH with p-values *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

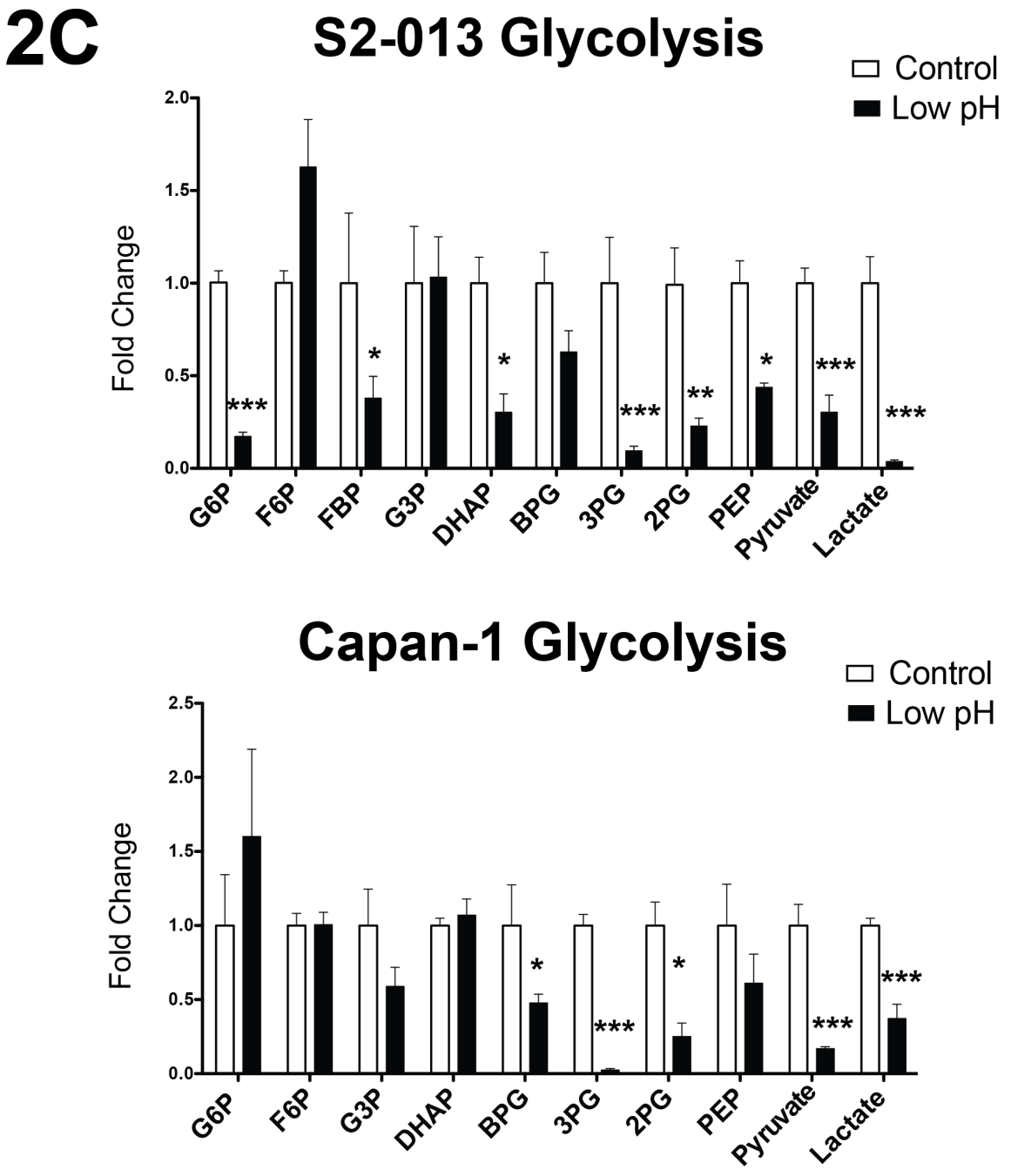


Figure 2C. PDAC cells in chronic low pH exposure shows a significant decrease in glycolysis. Polar metabolites were extracted from S2-013 and Capan-1 cells cultured in control and low pH. LC/MS/MS-based metabolomics was used to quantify glycolysis metabolites showing a significant decrease in glycolysis metabolic flux in low pH culture conditions.

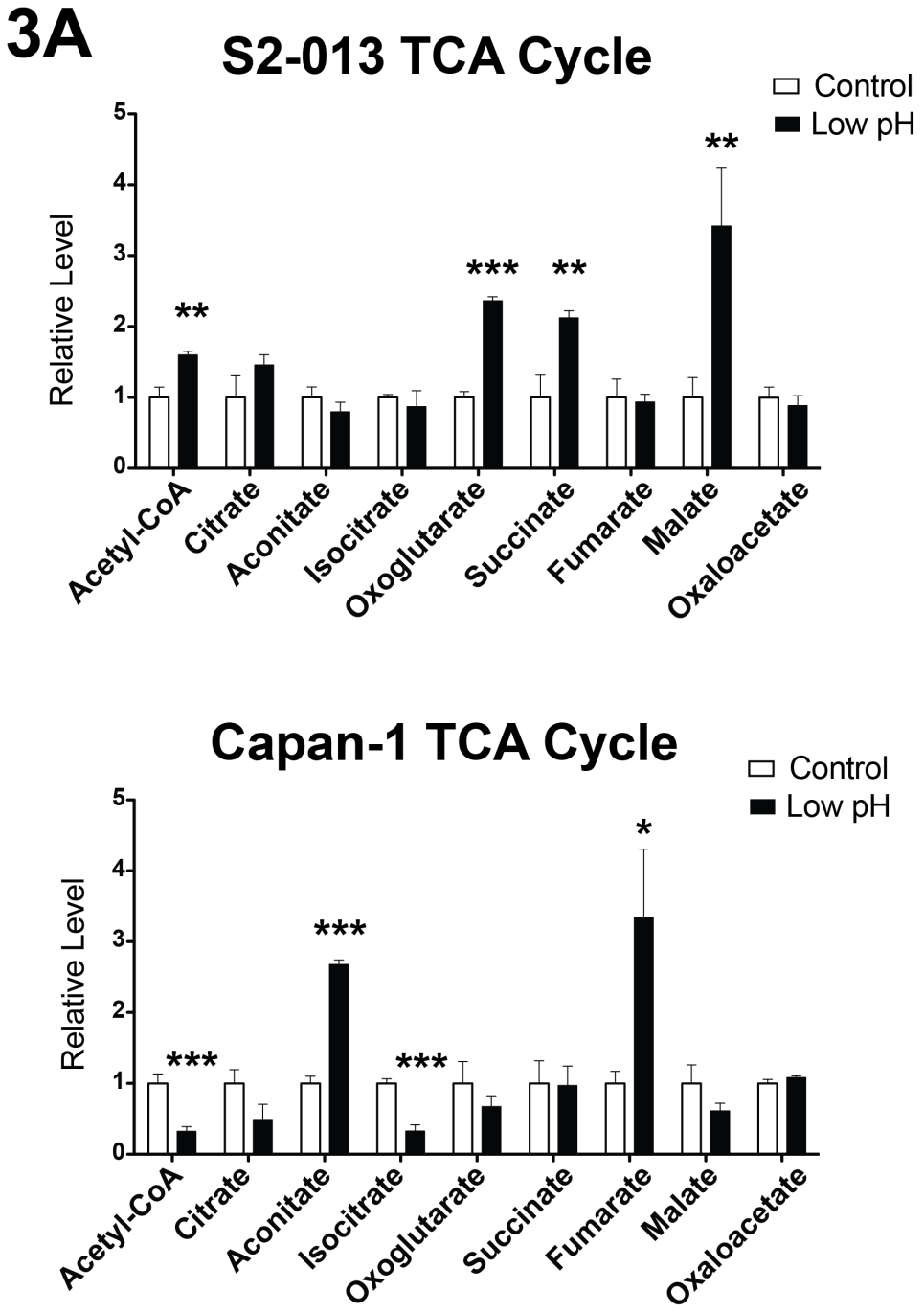


Figure 2C. TCA cycle metabolic flux is maintained in chronic low pH. Polar metabolites were extracted from S2-013 and Capan-1 cells cultured in control and low pH. LC/MS/MS-based metabolomics was used to quantify TCA cycle metabolites showing that unlike glycolysis TCA metabolism is not down regulated. Data in bar charts is normalized to the values for the control pH (7.4). Error bars represent mean ± S.E.M. from at least three different samples. A two-tailed Student's t-test to represent this data with p-values *p < 0.05, **p < 0.01, ***p < 0.001.

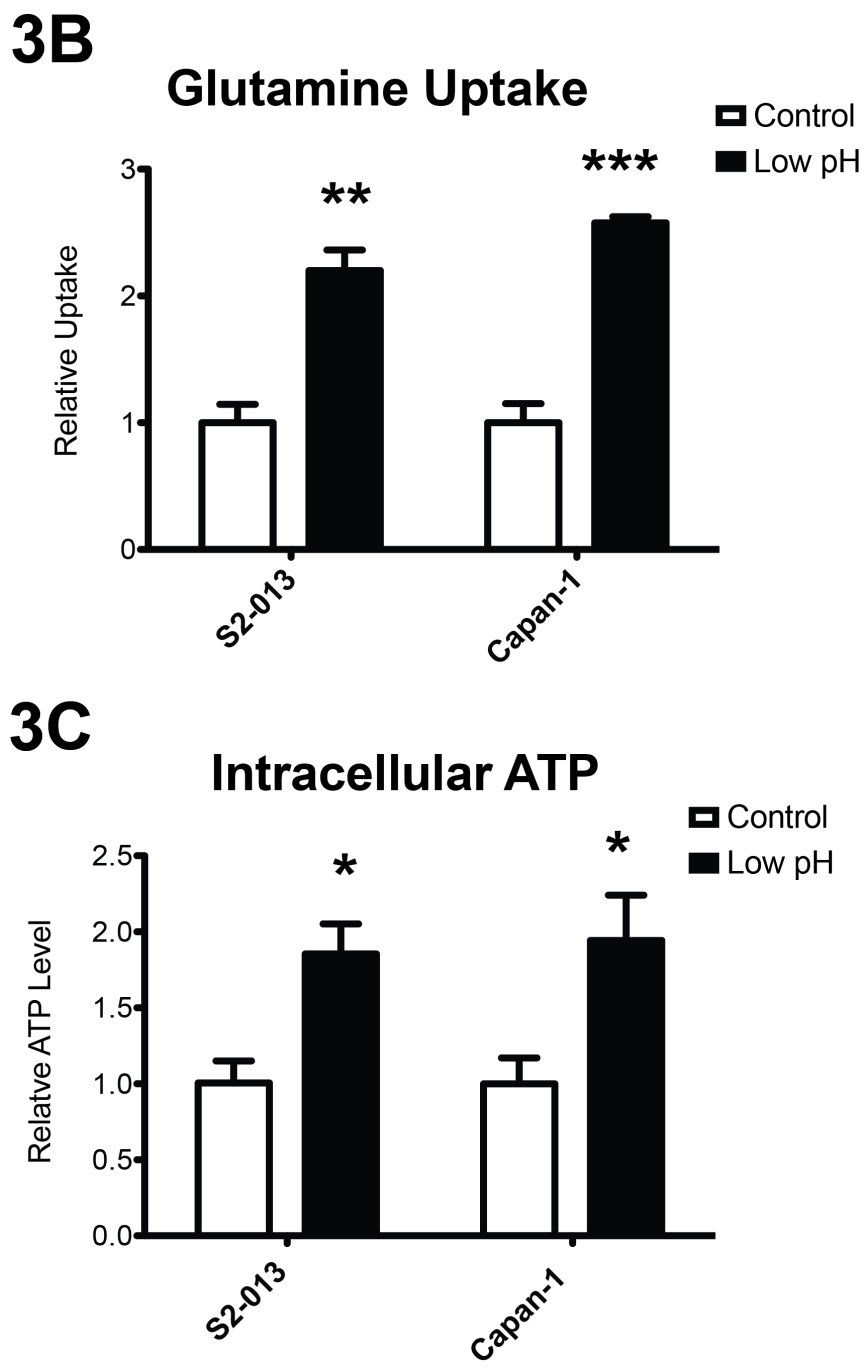


Figure 3B, 3C. PDAC cells in chronic low ph show a significant increase of glutaminolysis. Compared to control pH, PDAC cells in chronic low ph show a significant increase in glutamine uptake and intracellular ATP levels as shown by ^{3}H-glutamine uptake and bioluminescent assay to determine ATP levels in cell lysates. Error bars represent mean ± S.E.M. from at least three different samples. A two-tailed Student's t-test was conducted to compare these data sets with p-values *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3D

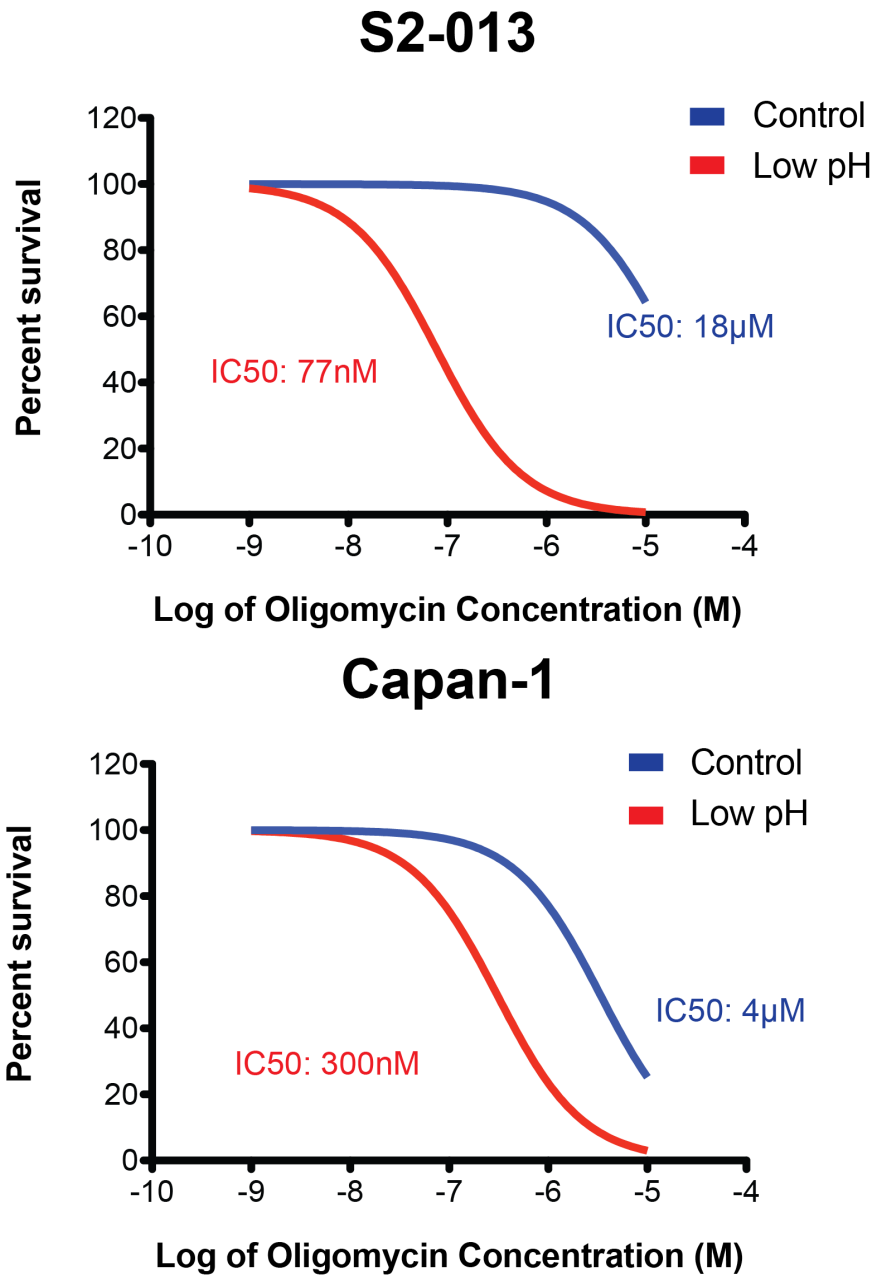


Figure 3D. PDAC cells in chronic low ph increase use of oxidative phosphorylation. Cells cultured in low pH show a marked decrease in survival upon oligomycin treatment. Oligomycin inhibits complex V of the electron transport chain.

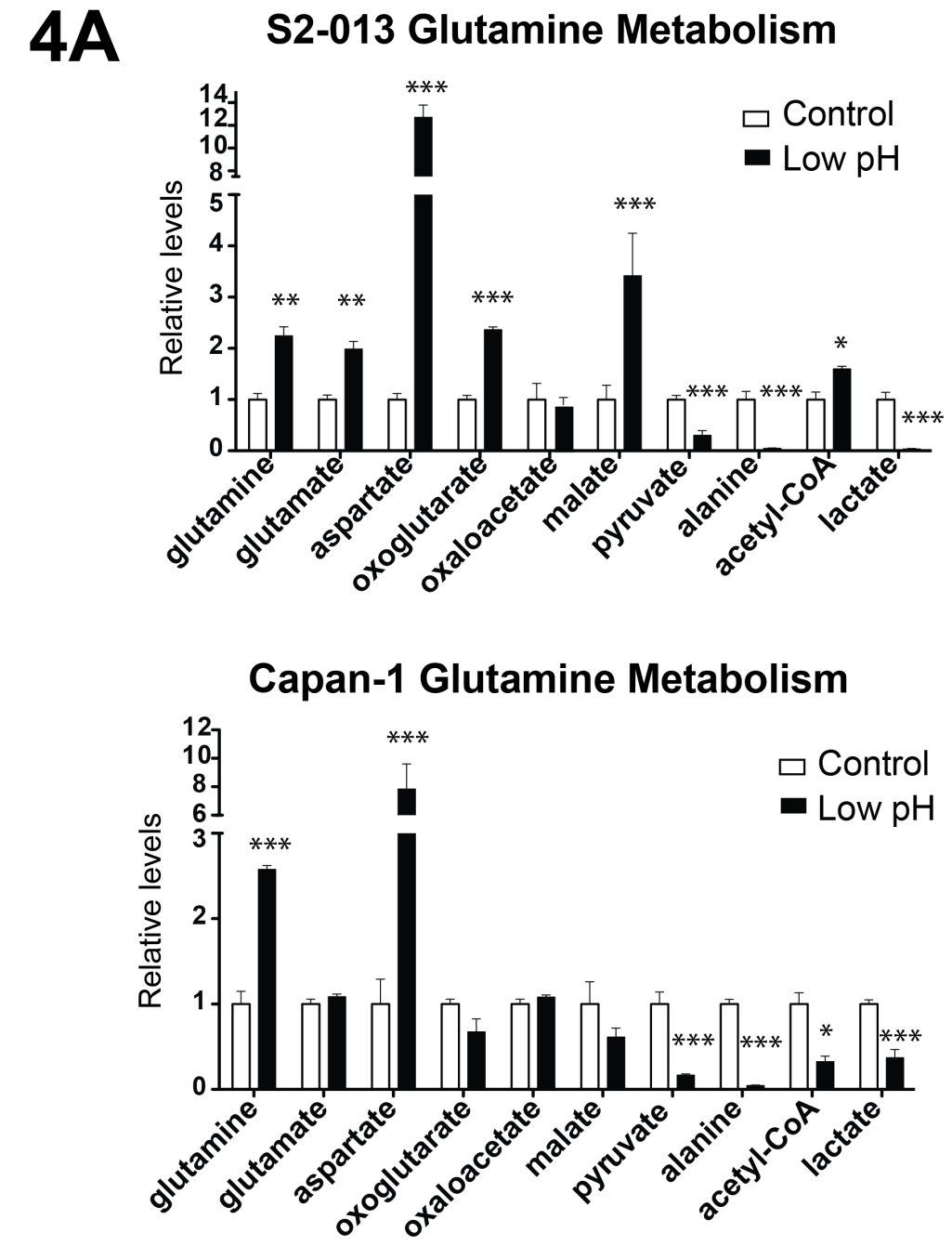


Figure 4 A. Metabolic flux through non-canonical anaplerotic glutamine metabolism is enhanced in chronic low ph. (A) LC/MS/MS-based metabolomic analysis of non-canonical glutamine metabolism in control (7.4) and low pH (7.0) culture conditions shows increased metabolic flux through the glutamate transaminase-driven pathway. Data in bar charts is normalized to the values for the control pH (7.4). Error bars represent mean ± S.E.M. from at least three different replicates. A two-tailed Student's t-test was conducted to compare control versus low pH in Fig. 4A and B with p-values *p < 0.05, **p < 0.01, ***p < 0.001.

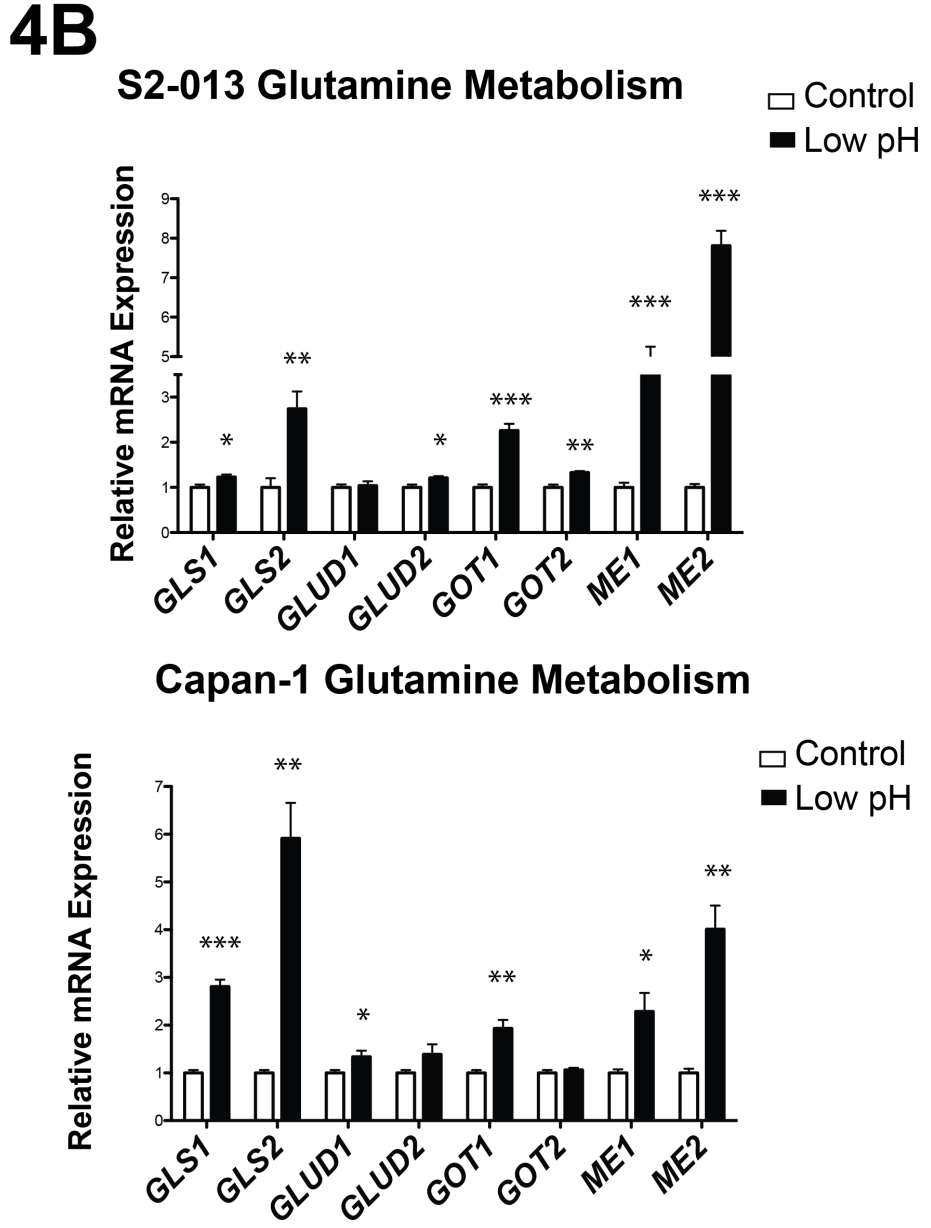


Figure 4 B. Transcription of enzyme mediators of non-canonical anaplerotic glutamine metabolism is increased chronic low ph. Quantitative real-time PCR analysis of genes coding for enzymes involved in non-canonical glutamine metabolism in cells cultured under control and low pH. Data shows an increase of transaminase enzymes and malic enzyme both of which are mediators of non-canonical glutamine metabolism. Data in bar charts is normalized to the values for the control pH (7.4). Error bars represent mean ± S.E.M. from at least three different replicates. A two tailed Student's t-test was conducted to compare control versus low pH in Fig. 4A and B with p-values *p < 0.05, **p < 0.01, ***p < 0.001.

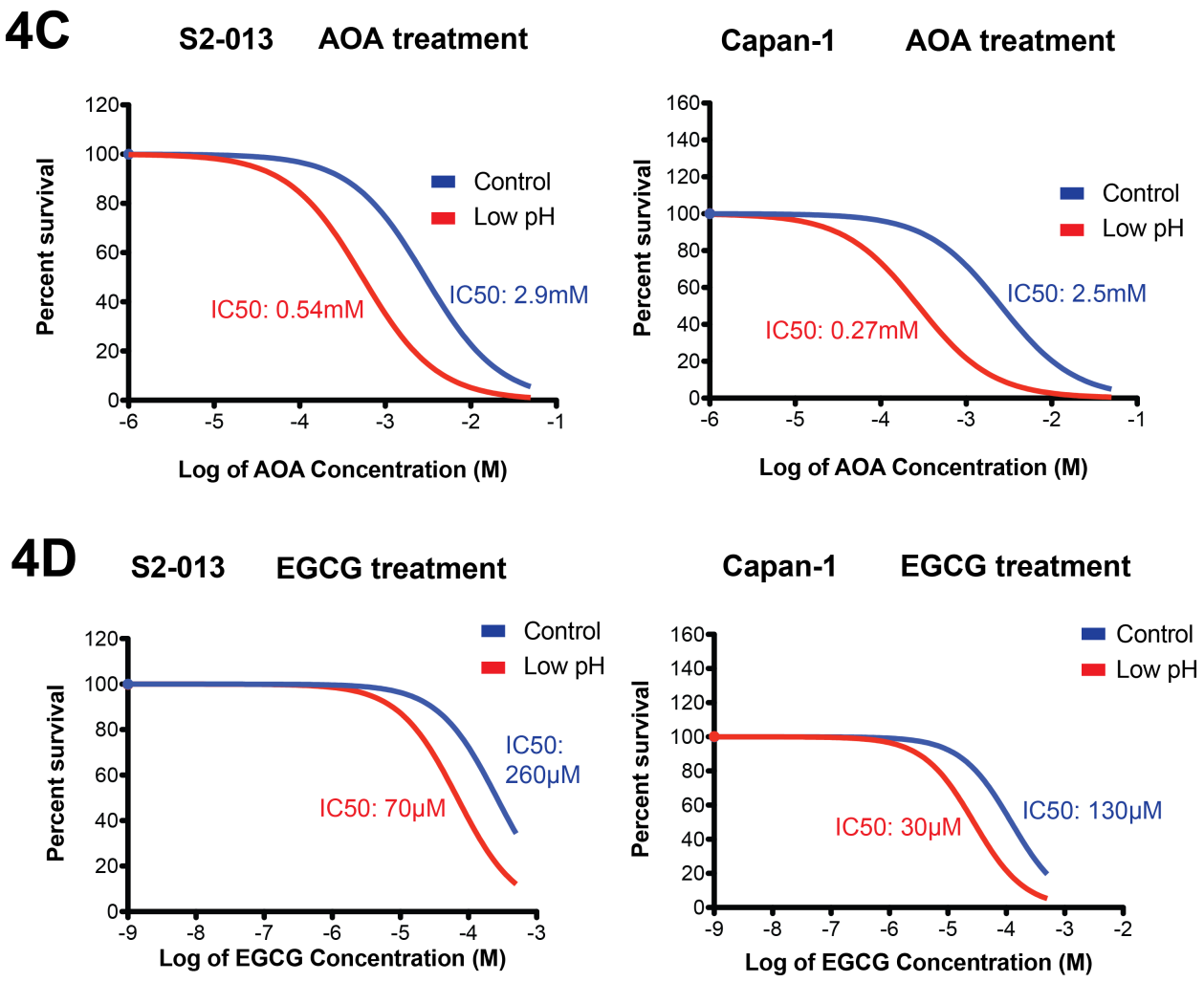


Figure 4C and 4D. PDAC cells cultured in low pH conditions are more sensitive to inhibition of glutamine metabolism. Treatment of S2-013 and Capan-1 cells to treatment with aminooxyacetic acid (AOA; C) and to epicogallocatechin gallate (EGCG; D) in control and low pH conditions shows increased sensitivity to glutamine metabolism. However, cells in low pH are more sensitive to inhibition of the anapleurotc pathway.

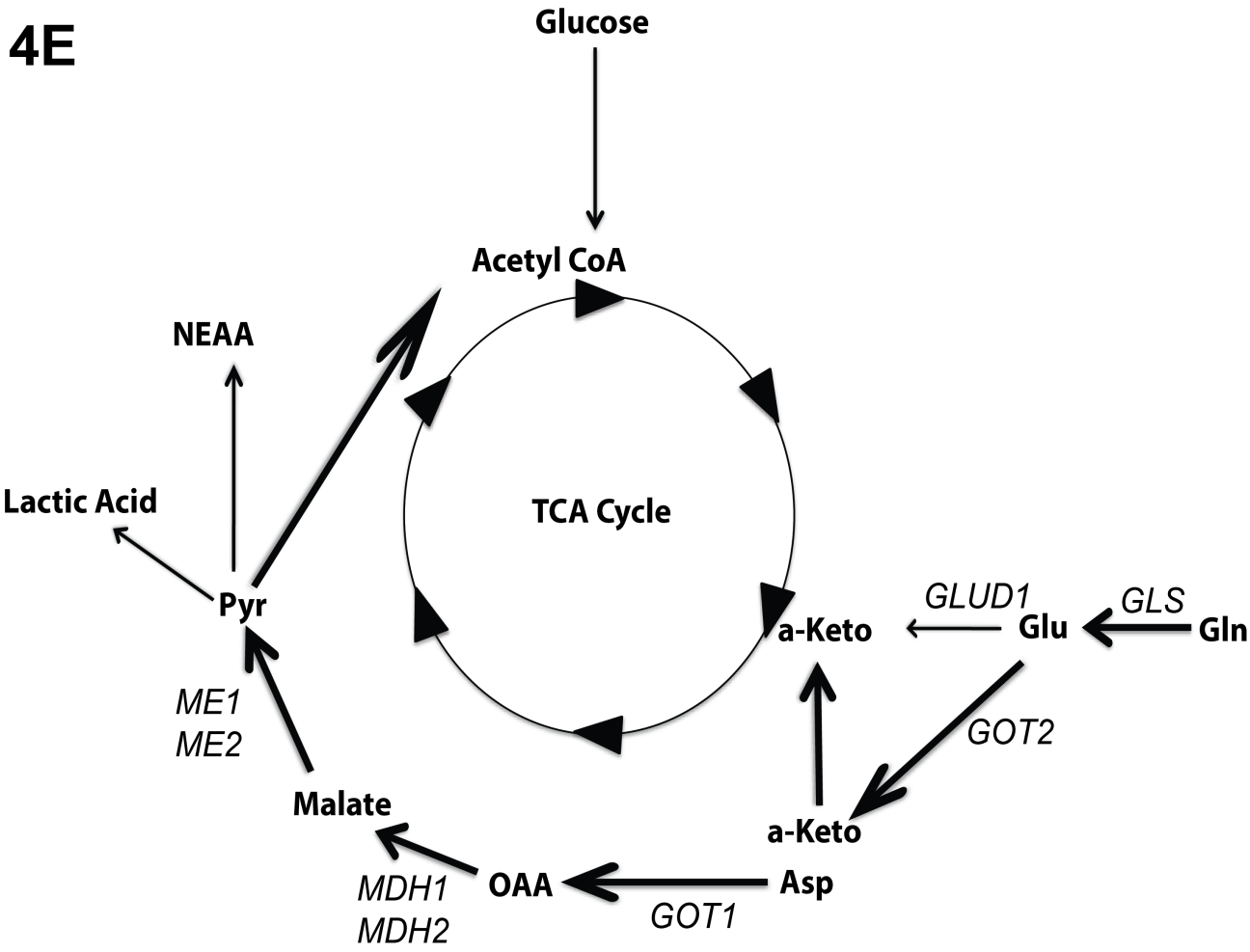


Figure 4E. PDAC cells cultured in low pH conditions are more sensitive to inhibition of glutamine metabolism. Figure 4E shows a schematic illustration of potential metabolite flow of glutamine in low pH culture conditions. The bold arrows denote the metabolic flux of glutamine through the non-canonical pathway. Glutamine is metabolized by glutamate synthase (GLS) and converted to glutamate, which is then metabolized by glutamate dehydrogenase (GLS1) and/or glutamic oxaloacetic transaminase 2 (GOT2). Subsequently, aspartate is metablized by glutamic oxaloacetic transaminase 1 (GOT1) to oxaloacetate, which is converted to malate and then to pyruvate by the malic enzymes. GOT1 expression is enhanced by oncogenic KRAS and Malic Enzymes are critical fro NADPH production for redox homeostasis.

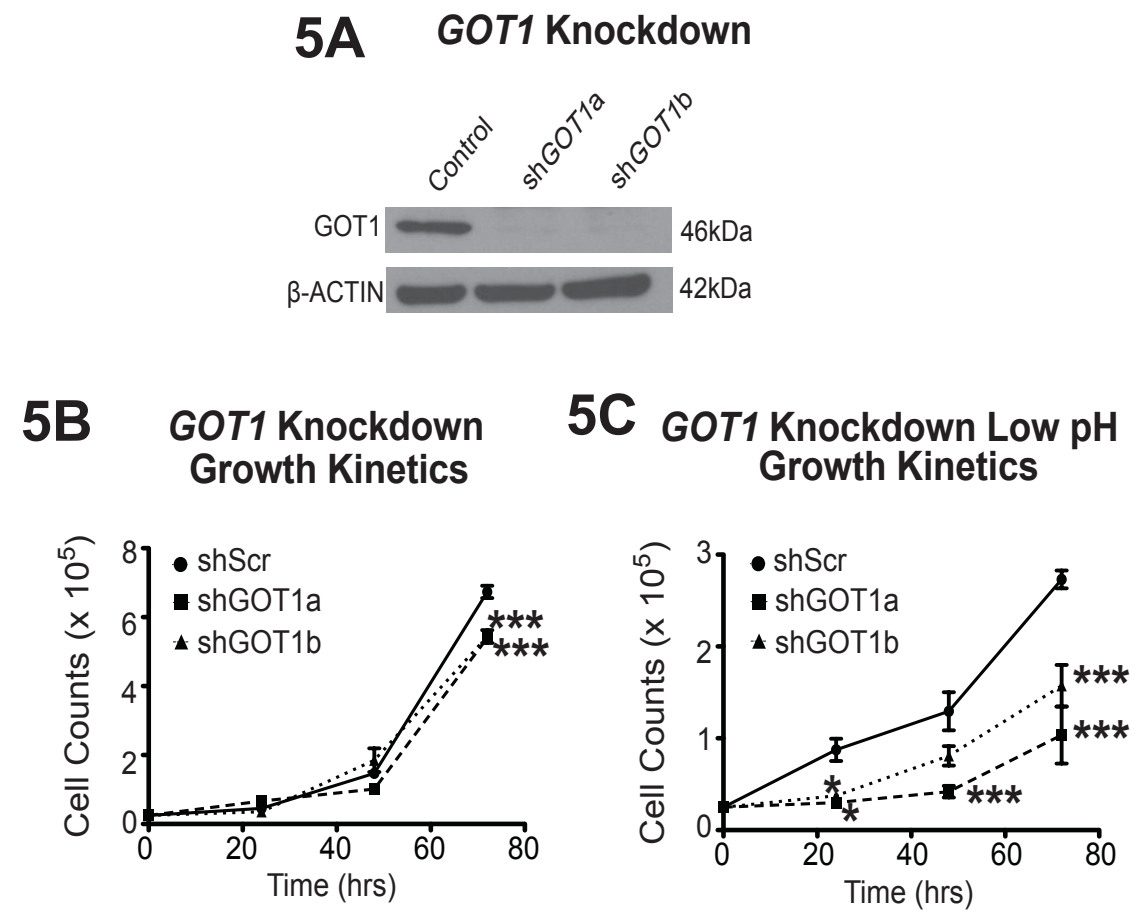
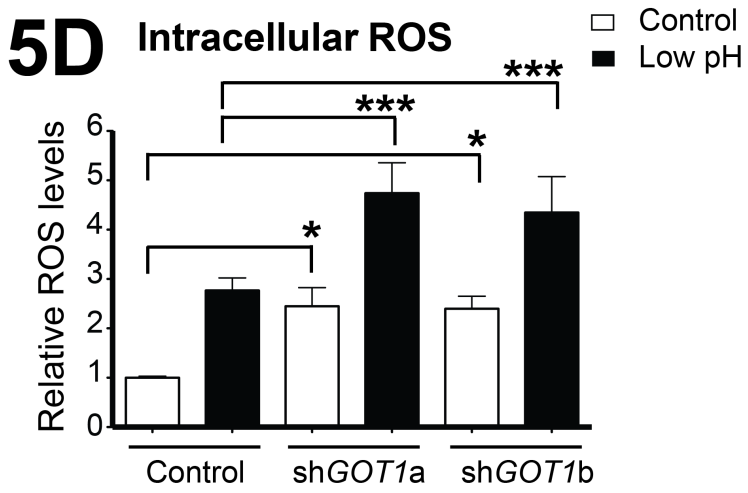


Figure 5A, 5B, and 5C. Knockdown of GOT1 inhibits growth of PDAC cells in low pH. (A) Western blotting to confirm the knockdown levels of GOT1 in S2-013 with two independent targets by utilizing lentiviral delivery. Cell growth of GOT1 knockdown and scrambled-control (shScr) cells in control pH (B) and low pH (C). GOT-1 knockdown significantly reduces cell growth in both culture conditions, but cells in low pH are severely impaired. A two-way ANOVA analysis, followed by Bonferroni posttests, was conducted to compare growth curves with p-values *p < 0.05, **p < 0.01, ***p < 0.001.



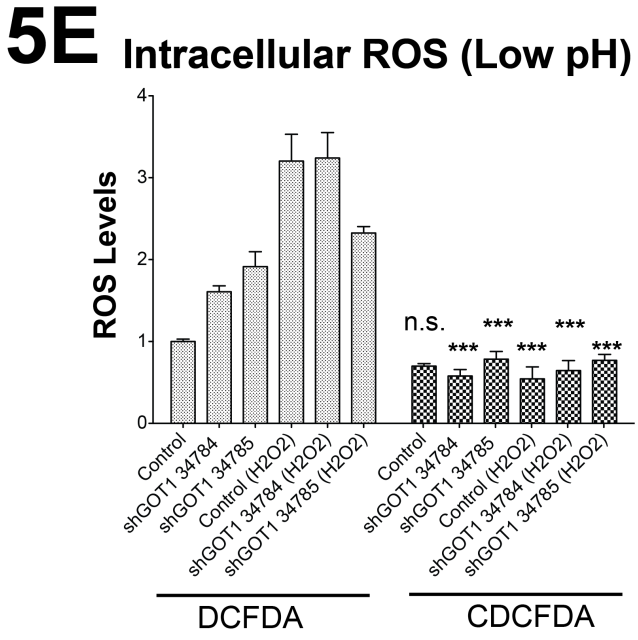


Figure 5D and 5E. GOT1 depletion further increases intracellular ROS levels in low pH culture. Fig. 5D shows measurement of intracellular ROS using carboxy-H_2DCFDA in control and low pH showing that ROS levels are higher in low pH culture. Furthermore, GOT1 knockdown leads to increase ROS levels in both control in low pH, but increase of intracellular ROS is much greater in low pH knockdown cells. Fig. 5E shows the measurement of intracellular ROS of cells in low pH by staining with carboxy-H_2DCFDA (DCFDA) and staining with CDCFDA, a ROS-insensitive dye. The ROS-insensitive compound was used to show fluorescence was not an artifact of cell culture using hydrogen peroxide treatment as a positive control as it induces ROS. The experiment shows that CDCF stain does not detect ROS levels. Data in bar charts is normalized to the values for the control pH (7.4). A two-way ANOVA analysis, followed by Bonferroni posttests, was conducted to compare different treatments represented on all the other panels with p-values *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

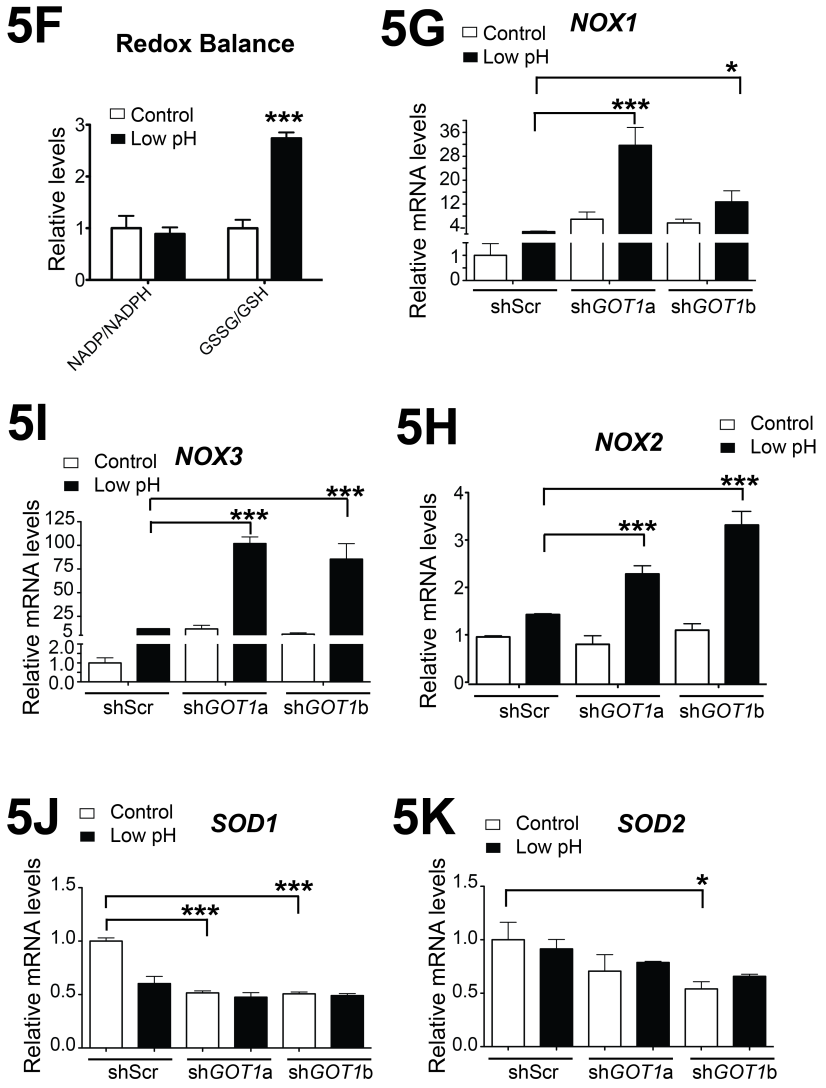


Figure 5F-5K. GOT1-mediated anaplerotic glutamine metabolism produces NADPH for ROS homeostasis. Fig. 5F LC/MS/MS measurement of NADP/NADPH and GSSG/GSH ratios in control and low pH culture shows similar NADP/NADPH ratio, but the GSSG/GSH ratio is higher in low pH culture indicating deregulation in NADPH mediated ROS suppression. Figures G to K show quantitative real-time PCR analysis of genes coding for enzymes involved in ROS regulation through oxidation of NADPH. Here it is observed that in low pH transcription of NADPH oxidases (NOX) increases. NOX enzymes oxidize NADPH producing superoxide as a by-product and super oxide dismutase (SOD) removes super oxides by combining it with protons and producing hydrogen peroxide, which is then removed by reduced glutathione (GSSG-ox or GSH-red) through GSH peroxidase or catalase, which is unchanged in low pH (data not shown). This data implies ROS suppression occurs is of high priority in low pH. Data in bar charts is normalized to the values for the control pH (7.4). A two-way ANOVA analysis, followed by Bonferroni posttests, was conducted to compare different treatments represented on all panels with p-values *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

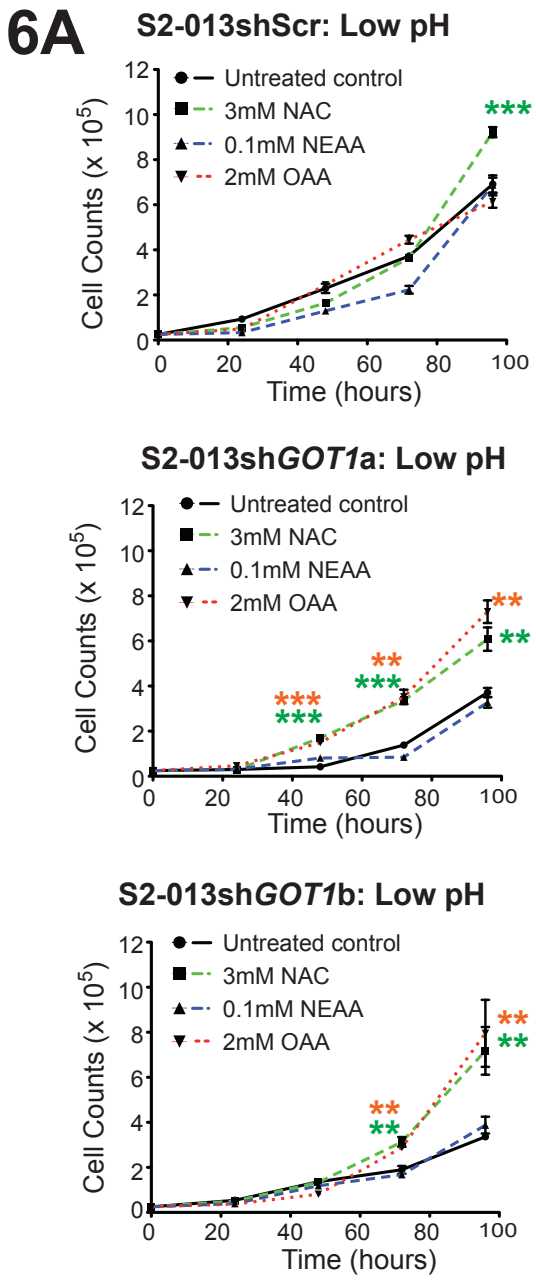
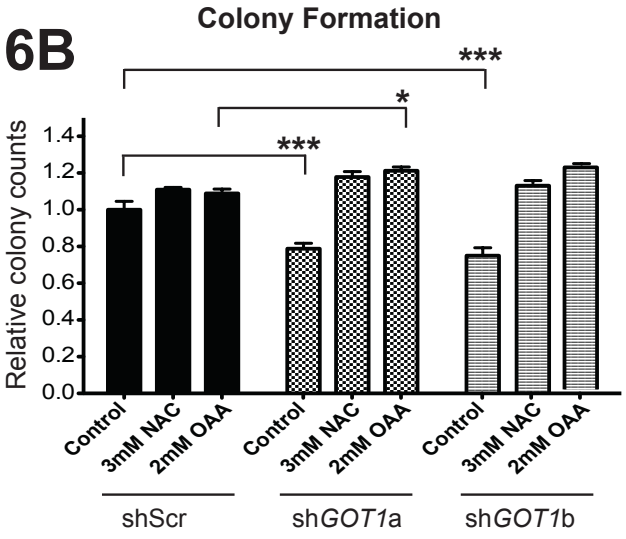


Fig. 6. Non-canonical glutamine metabolism regulates growth in chronic low ph. Growth kinetics of scrambled control, and GOT1 knockdown cells cultured in low pH media supplemented with N-acetyl cysteine (NAC), non-essential amino acids (NEAA), or oxaloacetate (OAA). OAA is the product of the reaction carried by GOT1. This experiment shows that growth is of knockdown cells in low pH is rescued by supplementing OAA and ROS-quencher NAC suggesting the significance of metabolic flux through GOT1 for ROS homeostasis. A two-way ANOVA analysis, followed by Bonferroni post-tests, was conducted to compare different treatments with p-value *p < 0.05, **p < 0.01, ***p < 0.001.



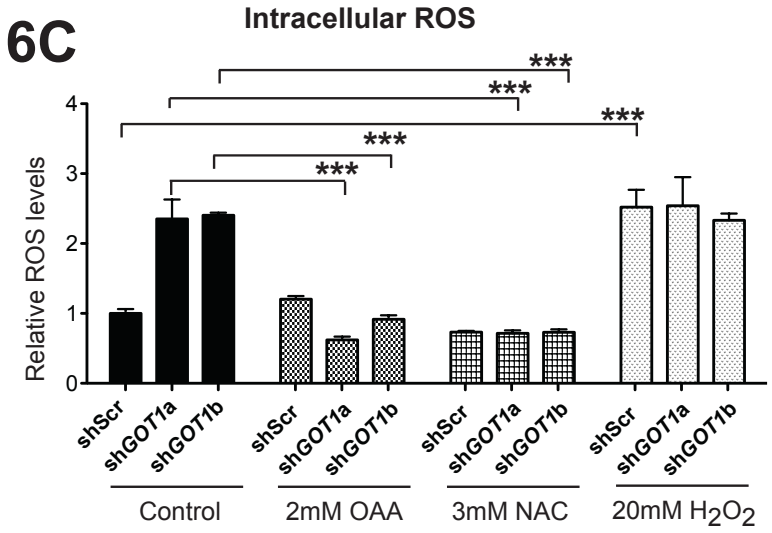


Figure 6B and 6C. Non-canonical glutamine metabolism regulates ROS levels in chronic low ph. Fig. 6B colony formation of control and GOT1 knockdown cells supplemented with NAC and OAA shows rescue of clonogeneicity upon pathway rescue (OAA) and ROS quenching (NAC). Fig 6C shows suppression of intracellular ROS levels in control and GOT1 knockdown cells supplemented with OAA and NAC, using H2O2 as a positive control. Data in bar charts is normalized to the values for untreated scrambled control. Error bars represent mean ± S.E.M. from at least three different replicates. A two-way ANOVA analysis, followed by Bonferroni post-tests, was conducted to compare different treatments with p-value *p < 0.05, **p < 0.01, ***p < 0.001.

Chapter 3

Discussion:

Conclusions & Future Directions

Summary of Work

Summary of Thesis:

Upon malignant transformation, PDAC cells modify their environment through activation of PSCs leading to the desmoplasia and the collapse of normal tissue vasculature that is replaced by the disorganized tumor vasculature. These changes in TME favor growth of cancer cells as the hypoxic microenvironment activates HIF inducing metabolic reprogramming that facilitates growth through up-regulation of glycolysis. Similarly, oncogenes such as KRAS promote increased uptake of glucose and glutamine, which are metabolized to produce energy, biomass, and co-factors for redox reactions. Collectively, HIF activation and oncogenes cause Warburg effect metabolism that is characterized by increased glucose uptake and its conversion to lactate and protons, glutamine can be converted to lactate as well. The increased metabolic rate of cancer cells results in accumulation of protons in the TME. Interestingly, consistent measurements of intracellular and extracellular pH of growing cancer cells show that pH_i is alkaline and pH_e is acidic. Decrease in pH_i has been shown to result in growth inhibition and apoptosis, while acidic pH_e has been shown to promote acidosis through activation of extracellular proteases that degrade ECM. Based on these observations, the role of acidosis in carcinogenesis is being redefined from a collateral effect of up-regulated metabolism to a hallmark of cancer. However, the disorganized vasculature of tumors becomes an inefficient method to vent metabolic acids from the TME. This creates a paradox as cancer cells require a high metabolic rate to proliferate, but further disparity in the proton gradient between the cytosol and the extracellular space of cells, which will prevent efficient proton efflux from the cytosolic compartment thereby lowering pH_i. Cancer cells cannot

withstand cytosol acidification because excess protons will affect the ionization of protonatable residues of amino acids in proteins thereby affecting their normal function. Thus, maintenance of the aforementioned pH gradient is critical for survival of cancer cells. For this reason cancer cells have developed complex mechanisms to regulate pH through active transport of charged molecules across the plasma membrane. Based on the pH challenge PDAC cells experience in the TME, I hypothesized that further survival of PDAC cells is through selection of mechanisms allowing cells to regulate metabolism or reprogram their metabolism in order to satisfy pH homeostasis. To this end I replicated chronic acidification similar to tumor conditions and demonstrated that chronic low ph causes growth inhibition likely due to increased ROS levels. In this low pH_e conditions, it was demonstrated that cells survive by reprogramming their metabolism by enhancement of anapleurotic glutamine metabolism at the expense of reducing glycolytic rate. The pathway is enhanced by oncogenic KRAS expression and it serves the purpose of generating energy for cellular homeostasis, as well as, providing the cells with NADPH for redox control.

Conclusions based on experimental evidence and extant literature:

In vivo venting of protons depends on the proximity of cells to blood vessels where the blood buffer system can remove excess protons. Therefore, the TME of PDAC will contain cells far away from the blood vessel where cells survive by activation of HIF response, which will cause an increase in dumping of protons onto the extracellular space. Absolute oxygen deprivation will kill the cells; thus, cells must be selected to move to gain oxygen access. At the same time, cells in the most hypoxic or oxygen deprived region will secrete excess protons into the extracellular space and the slow movement of protons toward the blood vessel will create the most

acidic region of the tumor. Cells in the most acidic part of the tumor will have to reprogram their metabolism in order to produce less protons, as their capacity to vent protons from the cytosol will be reduced by the increased extracellular proton gradient. Thus, a metabolic switch in which production of CO_2 is increased will be more favorable because CO_2 can passively diffuse across the plasma membrane. This metabolic shift is likely supported by several mechanisms, including the expression of nuclear respiratory factor 1, NRF1, that enhances mitochondria function, increase in expression of the isoform of cytochrome c oxidase subunit 4 isoform 1 change to isoform 2, COX4I1 to COX4I2, and mitochondria cristae remodeling to optimize ETC efficacy in low oxygen conditions [197, 198]. In this way, cells that can adapt to acidosis of TME can survive by generating CO_2 instead of protons. Cells that are more proximal to blood vessels, are driven to grow by increased expression of oncogenes, loss of tumor suppressors, and abundance of nutrients. Thus, this cell population experiences less selective pressure, but at the same time it bears the cancer phenotype of uncontrolled growth that will lead to continuous acidification of extracellular space. Thus, the cells closer to the nutrients exhibit Warburg effect and their release of protons into the TME will decrease venting of protons produced by cells further away from blood vessels. It is a common understanding that the most aggressive cells in PDAC tumors develop in the most hypoxic region, which is likely to be the most acidic as well [136]. **Figure 1** summarizes these statements.

Role of TME acidosis in carcinogenesis

Metastasis

In the past two decades the correlation between acidic TME and metastasis gained much needed interest leading to extensive studies of this relationship. Thus, it

has been shown that decreased pH_e promotes invasiveness by enhancing cytoskeletal dynamics leading to modification of cancer cell polarization, as well as, increase in proteolytic activity of TAMs and fibroblasts, as well as, activating ECM proteases released by the cancer cells themselves [34, 199, 200]. Interestingly, the contrasting alkaline pH_i promotes cytoskeleton remodeling by enhancing the activity of several acting binding proteins [201]. These observations were shown in vivo using intravital microscopy by monitoring HCT-116 cells, which showed that the lowest pH_e regions of the tumor underwent more cytoskeleton remodeling and had the highest expression of NHE1 in order to maintain alkaline pH_i [201]. Activation of proteases by acidic pH_e was demonstrated to increase angiogenesis by liberating pro-angiogenic factors trapped in ECM—at the same time this promoted metastasis of cancer cells by clearing path to the vasculature [202]. Another feature of TME acidosis that has gained interest over the years is the increase of stem cell markers in cells cultured in acidic pH medium [203]. Acid-induced stemness is not limited to cancer cells, as it has been demonstrated in osteosarcoma and melanoma that the low pH of the TME increases the population of mesenchymal stem cells, which contribute to tumor growth by secreting pro-tumorigenic factors [204]. Based on these observations, the process of carcinogenesis requires acidification of the TME, as it will continue selecting the most aggressive malignant phenotype. In addition passive CO_2 and H^+ venting of protons and active transport of protons, Gillies et al. demonstrated that the lysosme associated membrane protein 2, LAMP2, is critical for breast cancer tumorigenesis because it relocates to the plasma membrane to aid the process of TME acidification thereby enhancing metastasis [205]. These lines of experimental evidence highlight selection of acidic TME to further promote carcinogenesis.

Acidosis induces autophagy

Autophagy is a regulated cellular process through which dispensable organelles and macromolecules are turned over for metabolic purposes within the same cell . Pancreatic cancer cells show elevated autophagy under basal conditions and its inhibition leads to increased ROS, elevated DNA damage, and oxidative metabolic defects associated with mitochondria dysfunction [206]. As a result, inhibition of autophagy results in significant growth inhibition of PDAC cell lines, orthotopic mouse models, and genetic ablation of autophagy results in prolong survival in animal models. These observations suggest that autophagy is a feature of PDAC carcinogenesis, unlike other cancer types where autophagy is a last resource survival mechanism upon therapy-induced stress. Interestingly, increase in autophagy markers was demonstrated upon acidosis stress in breast cancer cell line MDA-MB-231 this phenotype was reversed/diminished in vivo upon administration of sodium bicarbonate [207]. The role of autophagy in acidosis stress was further elucidated in melanoma cells, which were unable to survive acidic stress upon knockdown of ATG5, autophagy marker necessary for the processes to take place [208]. Further studies have elucidated the role of pH_i acidification in inhibiting mTOR, which occurs by TSC complex acting as a pH sensor, protonation of residues inhibit its activation of mTOR, and by decrease in leucine uptake that activates mTOR mediated inhibition of autophagy [209, 210]. The role of mitophagy in acidosis may be just as essential for cell survival, as the mitochondria are critical for metabolic reprograming necessary to survive in low pH stress and functional mitochondria are required for metabolic reprogramming necessary to survive acidosis stress [211]. Similarly, functional autophagy requires the acidification of autolysosomes that achieve it through expression of V-ATPases allowing them to remove protons from the cytosol into the lysosome vesicle [212]. The transformation of autophagy from a tumor suppressive mechanism in normal cells into a pro-tumor mechanism in PDAC may

be a consequence of cancer cell pH dynamics. These observations further highlight the role of acidosis in PDAC carcinogenesis.

Acidosis of TME leads to immune suppression

Several lines of evidence show that acidification of the TME affects the anti-tumor immune response as exposure to lactate and protons decreases T-cell production of IL-2, IFNγ, perforin, and granzyme B, as well as, inhibiting TNF release by monocytes [213, 214]. Metabolomic analysis showed that low pH abrogates glycolysis in T-cells causing inhibition of cytokine synthesis. Similarly, it has been shown that the decreased immune response due to lowering pH_i prevents increased expression of nuclear factor of activated T-cells, NFAT, in NK cells and T-cells [215]. Studies showing the relationship between the metabolic rate of cancer cells and its effects on T-cells in the TME showed that T-cells deprived of glycolysis were unable to regulate NFAT-Ca^{2+} signaling to elicit an immune response [216]. This was denoted by the low levels of PEP that were corrected by overexpression of PEP carboxykinase, PCK, which allowed the cells to utilize oxaloacetate to make PEP and re-establish glycolysis [216]. Similar observations have been made upon ablation of LDHA, resulting in lower TME levels of protons and lactate. Thus, cancer cells in the TME inhibit immune response by outcompeting immune cells for nutrients and by exposing them to a low pH_e, which induces metabolic reprogramming to oxidative phosphorylation thereby inhibiting glycolysis-NFAT signaling that allows for production of cytokines. These observations further highlight the dependency of carcinogenesis in TME acidosis.

pH Sensing

It is estimated that the acid load in the extracellular space of tumors is up to 10 times higher than in the cytosolic compartment and can lead to pH change of up to 1 pH unit [217]. As I have previously described, excess protons are vented through the bicarbonate buffer system aided by blood and kidneys. However, it is predicted that long venting distances of more than 50μm will result in chronic low ph [217]. The most extensively studied pH sensors in the plasma membrane are G-protein coupled receptors, GPCRs, GPR4, OGR1, TDAG8, and G2A [218]. GPR4 stimulated by acidosis has been reported to activate the G_s $G_{12/13}$ pathways that increase cAMP levels and activate Rho-Gef pathways for induction of cell migration respectively [219]. Low levels of GPR4 in mouse models of melanoma results in impaired tumor growth and impairment of angiogenesis [218, 220]. Overexpression of OGR1 has been shown to inhibit metastasis in PC3 prostate cancer cells and in HEY ovarian cancer cells by increasing adhesion to several ECM proteins [218, 220, 221]. TDAG8 has been shown to increase tumor growth in Lewis lung carcinoma with similar findings shown in animal models using NCI-H460 non-small cell lung cancer cell line with knockdown of TDAG8 showing reduced survival in acidic conditions of tumor [219, 222]. TDAG8 activation by acidosis has been demonstrated to inhibit apoptosis upon nutrient starvation and its overexpression leads to transformation of mammary epithelial cells *in vitro* [219, 222]. Interestingly, in lymphoma high levels of TDAG8 has been shown to inhibit c-Myc expression thereby showing the ability of pH sensing to affect master regulators of metabolism [222]. G2A signaling is known to affect pH growth by inhabiting G2/M cell cycle progression resulting in mitosis inhibition [218]. Understanding the mechanisms of pH sensors will provide better insight into the molecular basis of pH-dependent survival of cancer cells in acidic TME by allowing us to understand their behavior in acidosis and eventually identify new diagnostics and therapeutics.

Although pH affects many cellular processes, one of the most sensitive systems in the cells is the actin cytoskeleton, which through its assembly and disassembly it determines vesicle trafficking, contraction, migration, invasion, and metastasis [57]. Interestingly, *de novo* trafficking assembly requires alkaline intracellular pH this process is greatly affected by decreasing the pH_i. This highlights the significance of alkaline pH_i as the cells will cease a lot homeostatic functions performed by the cytoskeleton if the pH drops. For example, cofilin requires the de-phosphorylation of a N-terminal serine and the deprotonation of a C-terminal histidine residue to de-polymerize filamentous F-actin and begin the process of de novo actin polymerization [223]. Thus, in cell trafficking cells need a higher cytosolic pH to re-assemble and dis-assemble actin filaments. Similarly, it has been demonstrated that the leading edge of focal adhesions in migrating cells has a higher pH_i this process is mediated by talin binding to F-actin in focal adhesions and that talin binding of F-actin filaments decreases if pHi drops bellow 7.2 [223]. This is due to protonation of amino acids that cause a conformational change in talin that prevents interatcition with F-actin filaments. Interestingly, binding of cofilin and GEFs with plextrin-homology domains to phosphoinositides has been shown to be pH dependent [143, 223]. A common feature of phosphoinositide recognition domains is that at lease one histidine is contained within the recognition domain this has not been shown experimentally, but proteins with this domain may be pH sensitive for membrane localization [143]. Thus, understanding the significance of pH-dependent histidine switches can help to understand pH dependent cytoskeleton function.

Targeting Ph_i Regulation For Therapy

TME acidosis creates a challenge in cancer therapy

Neutral molecules can pass freely through the plasma membrane, but the passage of charged compounds depends on extracellular pH—this phenomenon is known as ion trapping [57]. The acidic TME of tumors is unfavorable to weak base chemotherapeutic agents such as paclitaxel because they will be protonated. Thus, it is estimated that if the pH difference between the intra and extra cellular compartments is 1 pH unit there will be a ten-fold negative difference in weak base concentration in the cytosol [224]. Conversely, the more acidic chemotherapeutic agents such as cisplatin will localize to the alkaline intracellular environment of cells. These phenomena calls for intervention through proton pump inhibitors in order to control extracellular pH and predict better delivery of therapeutic agents. Neutralization of pH_e using systemic buffers such as $NaHCO_3$ has been proposed in efforts to identify which therapeutic modalities to take advantage of TME neutralization [225]. Indeed, bicarbonate treatment in hepatocellular carcinoma increases the efficacy of weak base chemotherapeutic agents [226]. Interestingly, this approach does not affect growth of the primary tumor, but it has been observed to reduce metastasis in animal models. Furthermore, bicarbonate treatment has also led to improved immunotherapy response by increasing response to anti-PD1 and anti-CTLA4 in melanoma mouse models [227]. The immunotherapy response can also be improved by inhibition of V-ATPases with proton pump inhibitors [228]. This observation was also shown in spontaneous mouse models of prostate adenocarcinoma where bicarbonate buffer treatment at 6-weeks of age, prior to tumor formation, resulted in lost of tumor formation and delay of tumor formation [229]. These studies highlight the significance of acidic pH_e in carcinogenesis. Addition of exogenous buffers may have adverse physiological effects, as it will lead to systemic pathologic alkalosis [225]. Therefore, instead of targeting the pH_e with buffers pH-sensitive drug delivery systems have been developed. These molecules include

peptides, liposomes, micelles, and polymeric nanoparticles [224, 230]. pH-low-insertion-peptides, pHLIPs, have been used to image PDAC tumor progression *in vivo* and *ex vivo* [231]. Thus, these molecules transport drugs to cancer cells through encapsulation or by chemical conjugation. These molecules work by having increased stability in physiological pH conditions and destabilizing or fusogenic in low pH environment [224, 230]. In this way, delivery to cancer cells in acidic TME is ensured and the viability of drugs is enhanced, as sequestration or inactivation during transport to tumor tissue will be diminished.

Inhibition of cancer pH_i regulation

Proton-pump-inhibitors (PPI) are pro-drugs that are activated by low pH generating sulfenaminde therefore in the acidic gastric environment or in TME they become activated and covalently interact with sulfhydryl groups of cysteine residues of V-ATPases inhibiting their activity [232]. These compounds have reached advance clinical trials as they have demonstrated to suppress tumor growth in mouse models and prevent acid-induced therapy resistance caused by ion trapping effect. Another target for therapy, are the carbonic anhydrases, CA, particularly CAIX and CAXII that are overexpressed in many tumor types compared to normal tissue [233]. Indeed, CAIX positive staining is an indicator of poor prognosis in PDAC [234]. Interestingly, CAs distribution in tumor tissues show increased expression of CAIX in the tumor edge and CAXII occurring more in the tumor center [235]. These observations have led to the development of specific CA inhibitors, which have shown to be potent growth inhibitors of primary tumor and metastases evidenced in mouse models. For this reason antibodies and small molecule inhibitors of CAs are currently undergoing clinical trials [236]. Monocarboxylate transporters, MCTs, which conduct the inward and outward transport of protons and lactate from cytosol to extracellular space, are

also a reasonable therapeutic target [237]. The MCT1,2 inhibitor AXD3965 has shown potent anti-tumor effects in mouse models and is currently undergoing clinical trials [238]. More advantageously is the development of inhibitors for hypoxia specific MCT4; to this end, it has been reported that the drug diclofenac inhibits lactate efflux in cancer cells independently of inhibition with cyclooxygenase [239]. Another obvious target for disruption of pH regulation in cancer cells is the inhibition of sodium hydrogen exchanger, NHE1, but no clinical break through has been achieved. This has been shown by knockdown of NHE1 and treatment with NHE1 selective pharmacological inhibitors amiloride and cariporide that show extensive anti-cancer effects *in vivo* [240]. However, side effects of pharmacological inhibition of NHE1 include myocardial infarction. Lastly, inhibition of sodium bicarbonate transporters, NBCs, is also a likely target to disrupt pH homeostasis. To his end, NBC specific compounds S0859 and S3705 have been used in vitro showing growth inhibition in 3D spheroid models and breast cancer cell lines [241].

Inhibition of pH regulation shows exceptional results in preventing further growth of tumors, but only a few compounds have reached clinical trials and they have delivered disappointing outcomes due to aberrant side effects. For example, MCT inhibitors are severely limited by the physiological role of the lactate shuttle in the brain or the overexpression of NHE1 in cardiac tissue [242, 243]. Another alternative is the dynamic nature of cancer, which upon selective pressure would lead to evolution of a compensatory mechanism with expression of different pH regulating proteins or reprogramming metabolism to produce less acids. To this end, new exiting data fro Grillo-Hill et al. shows that inhibition of proton cytosolic efflux in combination with up-regulation of oncogenic RAS expression is lethal for growing cells in Drosophila models [243]. These studies were also confirmed in cancer cells with

increased oncogene expression. Perhaps, a major break through could occur in the future with advancement in tumor imaging allowing for visualization of acidic regions of the tumor, which would make it easier to determine therapeutic approach.

Conclusion & Future Directions

Oncogenic KRAS drives metabolic response to acidosis

In chapter 1 of this thesis I've described the somatic evolution of pancreatic ductal cells into PDAC and concluded that malignant transformation is a slow process limited by tissue-mediated growth inhibition and dependent on metabolic substrate uptake. To this end, malignant cells gain oncogenic KRAS mutation and develop loss of function in tumor suppressors *TP53* and *CDKN2A*, which leads to unrestricted growth. The combination of intermittent hypoxia and oncogene expression in PDAC cells promotes growth and generation of an acidic TME. However, in order to sustain the cancer phenotype, PDAC cells must maintain alkaline pH_i and acidic pH_e pH dynamics, as deviation from this will be in detriment of growth and overall homeostasis of the cells. In order to regulate pH, cancer cells have developed active transport of protons into the extracellular space because increased pH_e will impair passive transport of metabolic acids. This creates a paradox for cancer cells, as further growth will be detrimental for pH balance. Thus, I hypothesized that further tumor progression depends on pH regulation and metabolic reprograming to meet homeostatic demands. In chapter 2, I conducted several experiments to determine the metabolism of PDAC cells in chronic low ph. Here, I identified that chronic low ph reduced proliferation rate and the cancer cells rely more on oxidative metabolism instead of glycolysis for survival. This metabolic phenotype is supported by increased glutamine uptake. Glutamine is metabolized through the non-canonical glutamine

anapleurotic pathway, which allows the cells to produce pyruvate for decarboxylation reactions and ATP synthesis through ETC, as well as, NADPH that is useful for redox balance. This pathway shows metabolic reprogramming under chronic low ph that will favor biomass production, redox balance, and energy production. Most significantly, this pathway is possible because oncogenic KRAS increases the expression of GOT1 and inhibits GLS1, which will lead to redirection of glutamine metabolism to the anapleurotic pathway. In non-acidosis conditions, the same pathway is essential for redox balance and growth, because NADPH production through anapleurotic glutamine metabolism compensates for lack of NADPH production from oxidative PPP. KRAS driven PDAC tumors are known to bypass oxidative PPP in order to accelerate rate of non-oxidative PPP to generate nucleic acids for cell replication. Therefore, oncogenic KRAS appears to be a critical regulator of cellular homeostasis in stress conditions by inducing metabolic reprogramming.

Future Directions

The observations by Grillo-Hill et al. confirm our hypothesis that up-regulated metabolic rate without means to regulate pH dynamics results in cell death. However, targeting pH regulation has been shown to be detrimental due to side effects of treatment, as all biological processes depend on pH regulation. Thus, one important avenue of research is to understand the signaling mechanisms allowing cancer cells to undergo metabolic reprogramming and membrane expression of pH regulating proteins. The pathways mediated by GPCRs and non-GPCR extracellular sensors have not been completely elucidated. Furthermore, known GPCR signaling is known cause inhibition of cytoskeleton remodeling not pH regulation per se. This is demonstrated by overexpression of GPR4 and OGR1 in cancer cells, which results in growth inhibition and invasiveness. This is likely because over-expression of pH

sensitive GPCRs will require less pHe disturbances to alert the cytosol of acidosis stress conditions and inhibit cytoskeleton remodeling [218]. While metabolic reprogramming and proton efflux would not be affected because the pH gradient is not changing; alternatively, the response can be induced by epigenetic changes as it has been demonstrated in HeLa cells that low pH_i favors histone de-acetylation and alkaline pH_i favors acetylation of histones [244]. Another interesting study to understand the evolution of pH dynamics in cancer is to evaluate somatic mutations, which occur randomly and may cause deleterious or advantageous alterations to gene function. However setting that gene product mutations aside, an interesting study regarding amino acid mutations landscape by Szpeech et al. shows that the predominant mutations in many solid tumors are that of arginine to histidine [245] [246]. These analyses predict that arginine→histidine mutations occur in nearly 50% of the samples analyzed. This mutation is of particular interest for pH_i regulation because of the role of histidine switches in actin remodeling control mechanisms due to acidic pH_i response. Furthermore, increase in histidine amino acid residues will result in an increase buffer capacity since the pK_a of histidine is around 6.5 and arginine is ~12, thus histidine will be able to titrate narrow pH changes [247]. Interestingly, p53 is one of the most studied molecules in cancer research because of its R273H mutation, which has been shown to be pH sensitive in a study by White et al. where it was demonstrated that arginine→histidine had increased transcriptional activity in low pH_i [246]. Thus, protonation of histidine will promote interaction with the phosphate backbone of DNA enabling transcription of p53 response genes. Further studies are required to elaborate more on the role of somatic mutations and their role in pH_i regulation, as well as, the pH-induced posttranslational modifications and subsequent phenotypic alterations. Based on the observations made throughout this document, future perspectives in cancer therapy should take in consideration the

biologic significance of pH dynamics in order to determine better therapeutic approaches.

Chapter 3 Figures

1

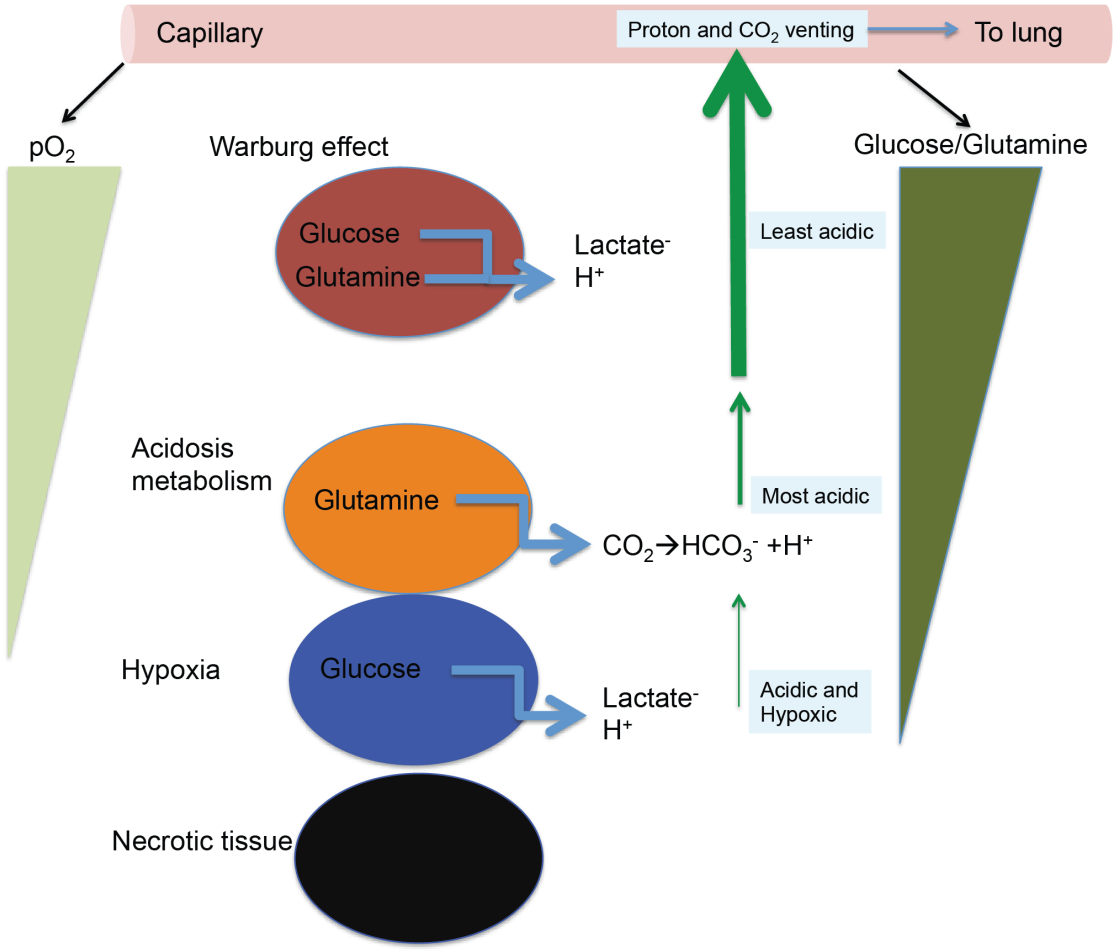


Figure 1. Hypothetic metabolic-pH relationship in PDAC tumors. The metabolism of PDAC cells is defined by the oncogenic KRAS and oxygen availability. PDAC cells that are proximal to blood vessels can undergo rapid growth because pH dynamics are not affected due to rapid venting of protons and CO_2. This is the classic Warburg effect seen during in vitro PDAC cell culture. Cells further away from blood vessels experience chronic low ph because proton venting is limited by growth of cells nearer to the vasculature. In addition, excess release of CO_2 and CA expression will convert it to carbonic acid, which may generate more protons and bicarbonate to buffer pH_i of cells undergoing acidosis stress. Cells that are the most distal from vasculature undergo hypoxia driven metabolic reprogramming that will further exacerbate acid load into the TME. Thus, cells closest to the hypoxic region will experience the most stress from TME acidosis.

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